Attorney Docket No. 044463-0336

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Vincent L. CHIANG et al.

Title: PRODUCTION OF SYRINGYL LIGNIN IN GYMNOSPERMS

Appl. No.: 10/681,878

Filing Date: October 9, 2003

Examiner: Stuart F. Baum

Art Unit: 1638

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

The undersigned, William H. Rottmann, hereby declares as follows:

1. I received my Ph.D. degree in Biochemistry in 1985 from the University of California, Berkeley. I have worked in the field of plant genetics since December 1984. Previously, I was a Senior Research Scientist at Westvaco/MeadWestvaco in Summerville, South Carolina. Currently I am a Senior Scientist at ArborGen and I am an expert in the area of gene regulation in wood development and flowering in trees. A copy of my resume is attached as Exhibit A.

2. I submit this declaration to establish that the promoter sequence claimed in the present invention is novel over the prior art and is adequately described in the specification. I additionally submit this declaration to establish that the specification of the present application provides extensive information to enable the person skilled in the art to make and/or use the claimed invention, because the sequence claimed in the present application comprises the necessary elements essential for promoter binding and activity.

3. I understand that the U.S. Patent and Trademark Office's position is that the Applicants of the present invention have not shown that the claimed 4CL1B promoter has promoter activity and have not specified the length and sequence of the promoter region which are essential for promoter activity.

4. I disagree with the U.S. Patent and Trademark Office's position. The promoter sequence claimed in the present application, SEQ ID NO: 11, corresponds to the 4CL1B promoter, which is described in Example 5 as being 2.3 kb in size. It is common usage among scientists to refer to genomic DNA fragments that comprise upstream regulatory regions and a portion of the 5'-untranslated region of the transcribed region as "promoter fragments". However, the 5'-untranslated region is not considered part of the promoter and can be eliminated in synthetic constructs to drive transcription of a different gene.

5. I assert that the cDNA sequence of a pine 4CL published by Voo *et al.* (*Plant Physiol. 108*: 85-95. 1995) can be used by one skilled in the art to verify that SEQ ID NO: 11 is a pine (and, therefore, gymnosperm) 4CL DNA sequence. The 100% identity found in the 142 base pair overlap with SEQ ID NO: 11 indicates that the two sequences are the same gene. Additionally, the cDNA sequence allows the skilled artisan to know where the 5'-untranslated sequence is. The promoter for the 4CL gene clearly lies upstream of base number 2107 of SEQ ID NO 11, where the match with the cDNA begins.

6. I further state that the cDNA sequence provided by Voo *et al* does not specify sequences sufficient for gene expression. I know of no examples where a plant cDNA sequence or segment thereof is sufficient to direct gene expression. In particular the CCAAT and TATA Box motifs, found upstream of the transcribed region, are missing from the cDNA sequence disclosed in Voo *et al.*

7. SEQ ID NO: 11 has two possible CCAAT-like motifs (positions 2018 – 2021 and 2014 – 2044) and two possible TATA motifs (positions 2037–2040 and 2066–2069) upstream of the 5'-untranslated region. The prior art does not provide these sites.

8. I further declare that since the 2001 filing date of this application, three different pine 4CL promoter fragments were tested in transgenic plants using the GUS reporter gene at ArborGen. Each of these promoter fragments showed xylem-preferred expression of the reporter gene. The first promoter fragment was the 2.3 kb sequence provided as SEQ ID NO: 11. The second promoter fragment was an approximately 1630 bp fragment of a Pinus radiata 4CL promoter with very high sequence similarity to SEQ ID NO: 11. The alignment of the 4CL1B promoter and the Pinus radiata 4CL promoter is attached herein as Exhibit B. The aligned sequences show great similarity in several regions and are over 95% identical in the segments corresponding to bases 630-1254 and 1404-2221 regions of SEQ ID NO: 11. The third promoter fragment was a 659 bp fragment of the same Pinus radiata promoter. The 659 bp Pinus radiata promoter fragment was 98.5% identical to the 659 bp fragment of SEQ ID NO: 10 disclosed in the present application.

9. A publication by Goicoechea *et al.* (*The Plant Journal 43*: 553-567 (2005)) is attached herein as Exhibit C. This publication shows that the MYB2 transcription factor isolated from *Eucalyptus gunnii* specifically binds to cis-regulatory regions in two lignin biosynthetic genes through a G(G/T)T(A/T)GGT(A/G) binding site.

10. I assert that all three pine 4CL promoter fragments and subfragments described above include the two GGTAGGTA motifs predicted to be MYB binding sites involved in promoter specificity according to Goicoechea *et al.* These motifs begin at position 1802 of SEQ ID NO: 11, and one of ordinary skill in the art would expect that DNA segments beginning after these motifs would not be able to specify xylem expression. These data confirm that the sequence claimed in the present application comprises the necessary elements essential for promoter binding and activity.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Date Mar 19, 2007 By William H. Rottmann

William H. Rottmann
Senior Scientist,
ArborGen, Summerville, SC

EXHIBIT A

WILLIAM H. ROTTMANN

PROFESSIONAL EXPERIENCE:

1/03 - present	Senior Scientist, ArborGen, Summerville, SC. (Gene discovery related to wood development in pine, eucalyptus, and cottonwood.)
10/98 – 12/02	Senior Research Scientist, Westvaco/MeadWestvaco, Summerville, SC. (Regulation of flowering in pine and cottonwood.)
11/95 - 10/98	Research Scientist, Westvaco, Summerville, SC. (Regulation of flowering in pine and cottonwood.)
2/91 - 10/95	Research Associate in the laboratory of Steven H. Strauss, Department of Forest Science, Oregon State University, Corvallis OR. (Isolation and characterization of cDNAs and genes expressed during development of cottonwood inflorescences.)
3/88 - 1/91	Research Geneticist in the laboratory of Athanasios Theologis, Plant Gene Expression Center, Albany, CA. (Isolation and characterization of cDNAs and genes from tomato that encode ACC synthase, a key enzyme in ethylene biosynthesis.)
12/84 - 1/88	National Science Foundation Postdoctoral Fellow in the laboratory of David M. Lonsdale, Institute for Plant Sciences Research (formerly Plant Breeding Institute), Cambridge, England. (Isolation and sequence analysis of mitochondrial gene responsible for male sterility in maize.)
8/78 - 12/84	Graduate Research Assistant in the laboratory of Edward E. Penhoet, Department of Biochemistry, UC Berkeley. (Isolation and sequence analysis of cDNAs and genes for human aldolases.)

EDUCATION:

Ph.D. 1985.	University of California, Berkeley. Biochemistry. Thesis title: Evolution of human aldolases.
B.S. 1978.	State University of New York at Stony Brook. Biochemistry (highest honors).

PEER-REVIEWED PUBLICATIONS:

Nehra, N.S., Becwar, M.R., Rottmann, W.H., Pearson, L., Chowdhury, K., Chang, S., Wilde, H.D., Kodrzycki, R.J., Zhang, C., Gause, K.C., Parks, D.W., Hinchee M.A. 2005. Forest Biotechnology: Innovative Methods, Emerging Opportunities. In Vitro Cell. Devel. Biol. – Plant 41: 701-717.

Rottmann, W.H., Meilan, R., Sheppard, L.A., Brunner, A.M., Skinner, J.S., Ma, C., Cheng, S., Jouanin, L., Pilate, G., and Strauss, S.H. 2000. Diverse effects of overexpression of *LEAFY* and *PTLF*, a poplar (*Populus*) homolog of *LEAFY/FLORICAULA*, in transgenic poplar and *Arabidopsis*. Plant J. 22: 235-245.

Brunner, A.M., Rottmann, W.H., Sheppard, L.A., Krutovskii, K., DiFazio, S.P., Leonardi, S., and Strauss, S.H. 2000. Structure and expression of duplicate *AGAMOUS* orthologues in poplar. Plant Molec. Biol. 44: 619-634.

Sheppard, L.A., Brunner, A.M., Krutovskii, K.V., Rottmann, W.H., Skinner, J.S., Vollmer, S.S., and Strauss, S.H. 2000. A *DEFICIENS* homolog from the dioecious tree *Populus trichocarpa* is expressed in both female and male floral meristems of its two-whorled, unisexual flowers. Plant Physiol. 241: 627-639.

Brunner, A.M., Mohamed, R., Meilan, R., Sheppard, L.A., Rottmann, W.H., and Strauss, S.H. 1998. Genetic engineering of sexual sterility in shade trees. J. Arbor. 24: 263-273.

Hipkins, V.D., Marshall, K., Neale, D.B., Rottmann, W.H., and Strauss, S.H. 1995. A mutation hotspot in the chloroplast genome of a conifer (Douglas-fir: *Pseudotsuga*) is caused by variability in the number of direct repeats derived from a partially duplicated tRNA gene. Curr. Genet. 27: 572-579.

Strauss, S.H., Rottmann, W.H., Brunner, A.M., and Sheppard, L.A. 1995. Genetic engineering of sterility in trees. Mol. Breeding 1: 5-26.

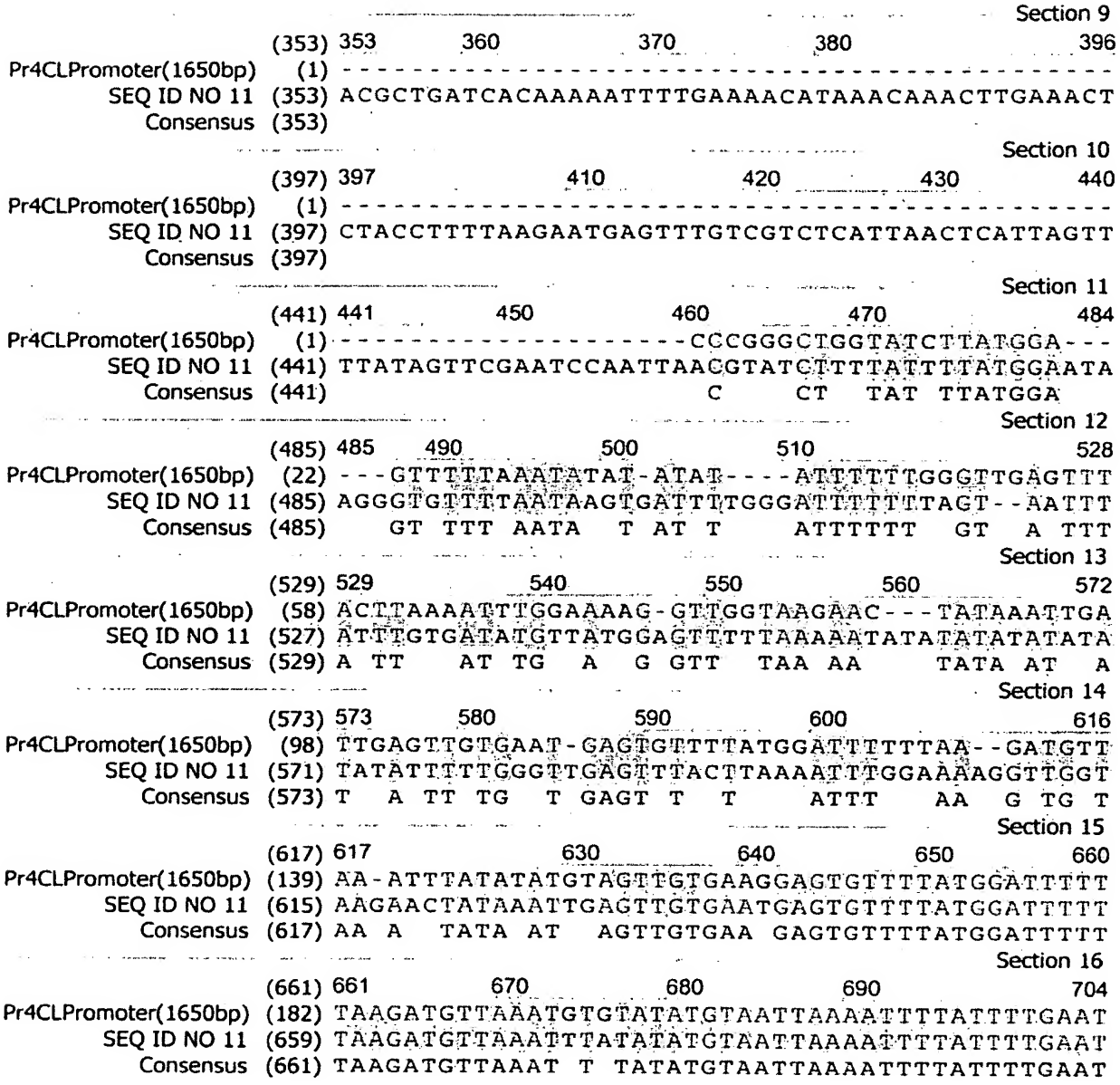
PATENTS AWARDED

US 6,444,877. Rottmann, W.H. 2002. Liquidambar styraciflua AGAMOUS (LSAG) gene.

US 6,395,892. Strauss, S.H., Rottmann, W., Brunner, A., and Sheppard, L. 2002. Floral homeotic genes for manipulation of flowering in poplar and other plant species.

EXHIBIT B

```
                                                                     Section 1
                        (1)  1        10        20        30            44
Pr4CLPromoter(1650bp)   (1)  --------------------------------------------
          SEQ ID NO 11  (1)  GGCCGGGTGGTGACATTTATTCATAAATTCATCTCAAAACAAGA
             Consensus  (1)
                                                                     Section 2
                       (45)  45   50        60        70                88
Pr4CLPromoter(1650bp)   (1)  --------------------------------------------
          SEQ ID NO 11 (45)  AGGATTTACAAAAATAAAAGAAAACAAAATTTTCATCTTTAACA
             Consensus (45)
                                                                     Section 3
                       (89)  89         100       110       120        132
Pr4CLPromoter(1650bp)   (1)  --------------------------------------------
          SEQ ID NO 11 (89)  TAATTATAATTGTGTTCACAAAATTCAAACTTAAACCCTTAATA
             Consensus (89)
                                                                     Section 4
                      (133)  133    140       150       160            176
Pr4CLPromoter(1650bp)   (1)  --------------------------------------------
          SEQ ID NO 11(133)  TAAAGAATTTCTTTCAACAATACACTTTAATCACAACTTCTTCA
             Consensus(133)
                                                                     Section 5
                      (177)  177          190       200       210      220
Pr4CLPromoter(1650bp)   (1)  --------------------------------------------
          SEQ ID NO 11(177)  ATCACAACCTCCTCCAACAAAATTAAAATAGATTAATAAATAAA
             Consensus(177)
                                                                     Section 6
                      (221)  221      230       240       250          264
Pr4CLPromoter(1650bp)   (1)  --------------------------------------------
          SEQ ID NO 11(221)  TAAACTTAACTATTTAAAAAAAAATATTATACAAAATTTATTAA
             Consensus(221)
                                                                     Section 7
                      (265)  265  270       280       290              308
Pr4CLPromoter(1650bp)   (1)  --------------------------------------------
          SEQ ID NO 11(265)  AACTTCAAAATAAACAAACTTTTTATACAAAATTCATCAAAACT
             Consensus(265)
                                                                     Section 8
                      (309)  309        320       330       340        352
Pr4CLPromoter(1650bp)   (1)  --------------------------------------------
          SEQ ID NO 11(309)  TTAAAATAAAGCTAAACACTGAAAATGTGAGTACATTTAAAAGG
             Consensus(309)
```


Section 9
(353) 353 360 370 380 396
Pr4CLPromoter(1650bp) (1) ----------
SEQ ID NO 11 (353) ACGCTGATCACAAAAATTTTGAAAACATAAACAAACTTGAAACT
Consensus (353)
Section 10
(397) 397 410 420 430 440
Pr4CLPromoter(1650bp) (1) ----------
SEQ ID NO 11 (397) CTACCTTTTAAGAATGAGTTTGTCGTCTCATTAACTCATTAGTT
Consensus (397)
Section 11
(441) 441 450 460 470 484
Pr4CLPromoter(1650bp) (1) ----------CCCGGGCTGGTATCTTATGGA---
SEQ ID NO 11 (441) TTATAGTTCGAATCCAATTAACGTATCTTTTATTTTATGGAATA
Consensus (441) C CT TAT TTATGGA
Section 12
(485) 485 490 500 510 528
Pr4CLPromoter(1650bp) (22) ---GTTTTTAAATATAT-ATAT----ATTTTTTGGGTTGAGTTT
SEQ ID NO 11 (485) AGGGTGTTTTAATAAGTGATTTTGGGATTTTTTTTAGT--AATTT
Consensus (485) GT TTT AATA T AT T ATTTTTT GT A TTT
Section 13
(529) 529 540 550 560 572
Pr4CLPromoter(1650bp) (58) ACTTAAAATTTGGAAAAG-GTTGGTAAGAAC---TATAAATTGA
SEQ ID NO 11 (527) ATTTGTGATATGTTATGGAGTTTTTAAAAATATATATATATATA
Consensus (529) A TT AT TG A G GTT TAA AA TATA AT A
Section 14
(573) 573 580 590 600 616
Pr4CLPromoter(1650bp) (98) TTGAGTTGTGAAT-GAGTGTTTTATGGATTTTTTAA--GATGTT
SEQ ID NO 11 (571) TATATTTTTGGGTTGAGTTTACTTAAAATTTGGAAAAGGTTGGT
Consensus (573) T A TT TG T GAGT T T ATTT AA G TG T
Section 15
(617) 617 630 640 650 660
Pr4CLPromoter(1650bp) (139) AA-ATTTATATATGTAGTTGTGAAGGAGTGTTTTATGGATTTTT
SEQ ID NO 11 (615) AAGAACTATAAATTGAGTTGTGAATGAGTGTTTTATGGATTTTT
Consensus (617) AA A TATA AT AGTTGTGAA GAGTGTTTTATGGATTTTT
Section 16
(661) 661 670 680 690 704
Pr4CLPromoter(1650bp) (182) TAAGATGTTAAATGTGTATATGTAATTAAAATTTTATTTTGAAT
SEQ ID NO 11 (659) TAAGATGTTAAATTTATATATGTAATTAAAATTTTATTTTGAAT
Consensus (661) TAAGATGTTAAAT T TATATGTAATTAAAATTTTATTTTGAAT

Section 17

(705) 705 710 720 730 748

Pr4CLPromoter(1650bp) (226) AACAAAAAATTATAATTGGATAAAAAAT-GTTTTGTTAAATTTA

SEQ ID NO 11 (703) AACAAAAA-TTATAATTGGATAAAAAATTGTTTTGTTAAATTTA

Consensus (705) AACAAAAA TTATAATTGGATAAAAAAT GTTTTGTTAAATTTA

Section 18

(749) 749 760 770 780 792

Pr4CLPromoter(1650bp) (269) GAGTAAAAATTTTAAAATCTAAAATAATTAAACACTATTATTTT

SEQ ID NO 11 (746) GAGTAAAAATTTCAAAATCTAAAATAATTAAACACTATTATTTT

Consensus (749) GAGTAAAAATTT AAAATCTAAAATAATTAAACACTATTATTTT

Section 19

(793) 793 800 810 820 836

Pr4CLPromoter(1650bp) (313) TAAAAAATTTGTTGGTAAATTTTATCTTAAATTTA-GTTAAAAT

SEQ ID NO 11 (790) TAAAAAATTTGTTGGTAAATTTTATCTTATATTTAAGTTAAAAT

Consensus (793) TAAAAAATTTGTTGGTAAATTTTATCTTA ATTTA GTTAAAAT

Section 20

(837) 837 850 860 870 880

Pr4CLPromoter(1650bp) (356) TTAGAAAAAAAAATAATTTTAAATTATTAAACTTTTGAAGTCAA

SEQ ID NO 11 (834) TTAGAAAAAAT--TAATTTTAAATTAATAAACTTTTGAAGTCAA

Consensus (837) TTAGAAAAAA TAATTTTAAATTA TAAACTTTTGAAGTCAA

Section 21

(881) 881 890 900 910 924

Pr4CLPromoter(1650bp) (400) ATATTCCAAATGTTTTCCAAAATATTAAATTCATTTGACATTCA

SEQ ID NO 11 (876) ATATTCCAAATATTTTCCAAAATATTAAATCTATTTTGCATTCA

Consensus (881) ATATTCCAAAT TTTTCCAAAATATTAAAT ATTT CATTCA

Section 22

(925) 925 930 940 950 968

Pr4CLPromoter(1650bp) (444) AAATACAATTTAAATAACAAAACTTCATGAAATAGATTAACCAA

SEQ ID NO 11 (920) AAATACAATTTAAATAATAAAACTTCATGGAATAGATTAACCAA

Consensus (925) AAATACAATTTAAATAA AAAACTTCATG AATAGATTAACCAA

Section 23

(969) 969 980 990 1000 1012

Pr4CLPromoter(1650bp) (488) TTTGTATGAAAACCAAAAATCTCAAATAAAATTTAAATTACAAA

SEQ ID NO 11 (964) TTTGTATAAAAACCAAAAATCTCAAATAAAATTTAAATTACAAA

Consensus (969) TTTGTAT AAAACCAAAAATCTCAAATAAAATTTAAATTACAAA

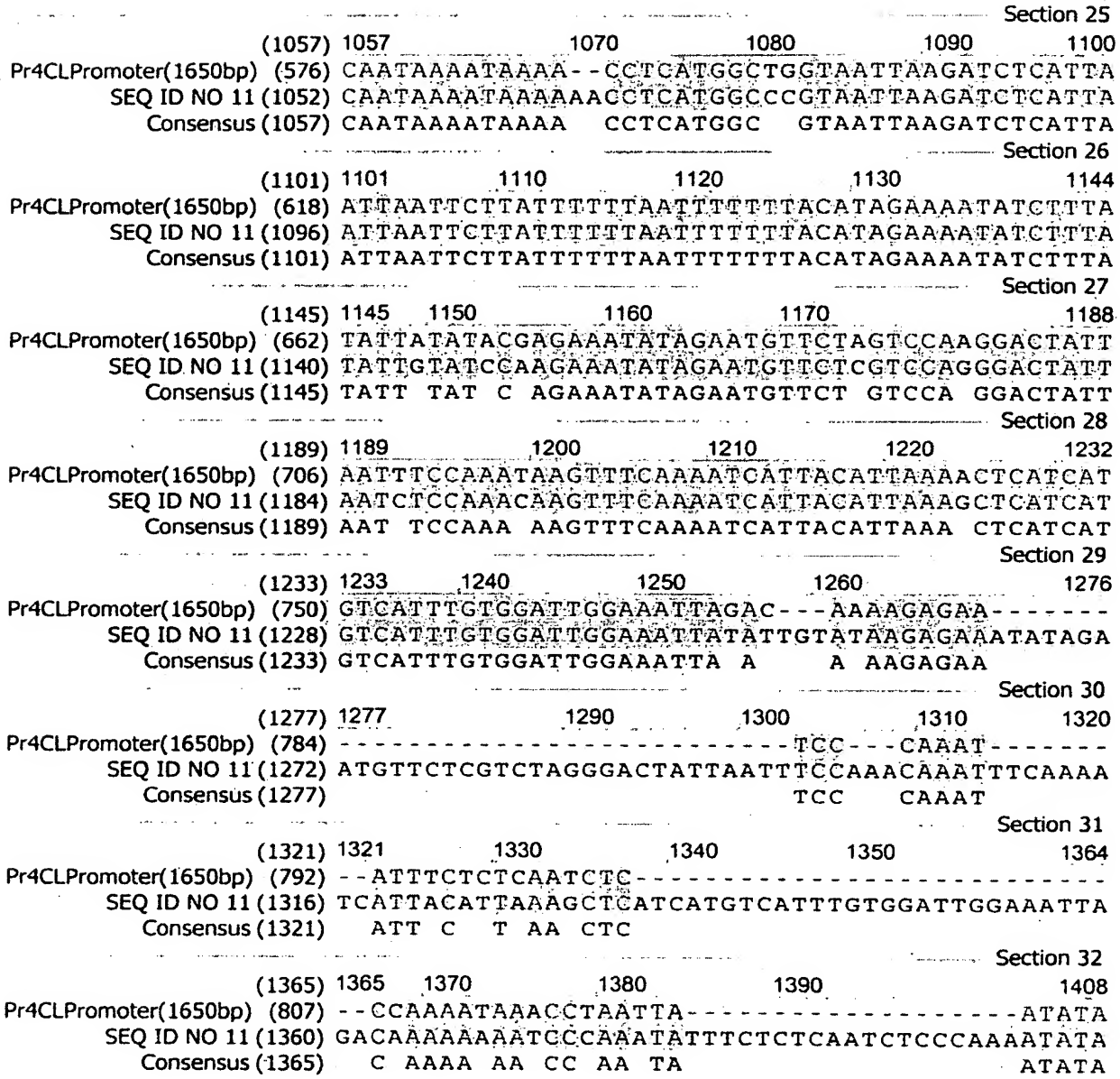
Section 24

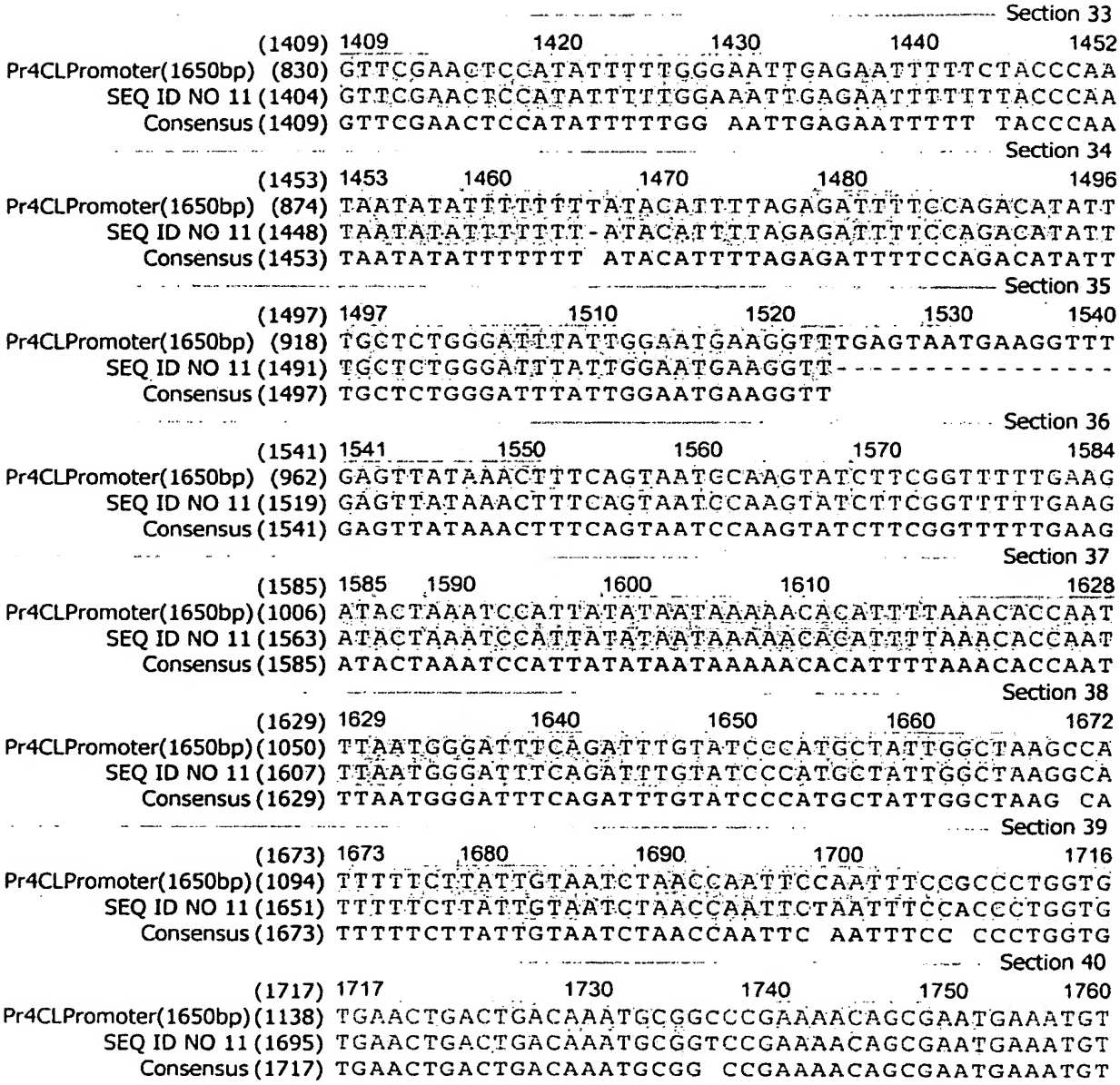
(1013) 1013 1020 1030 1040 1056

Pr4CLPromoter(1650bp) (532) ATATTATTAACATTATGATTTCAAGAAAGAGAATAACCAGTTTC

SEQ ID NO 11 (1008) ACATTATCAACATTATGATTTCAAGAAAGACAATAACCAGTTTC

Consensus (1013) A ATTAT AACATTATGATTTCAAGAAAGA AATAACCAGTTTC


Section 25
(1057) 1057 1070 1080 1090 1100
Pr4CLPromoter(1650bp) (576) CAATAAAATAAAA--CCTCATGGCTGGTAATTAAGATCTCATTA
SEQ ID NO 11 (1052) CAATAAAATAAAAAACCTCATGGCCCGTAATTAAGATCTCATTA
Consensus (1057) CAATAAAATAAAA CCTCATGGC GTAATTAAGATCTCATTA
Section 26
(1101) 1101 1110 1120 1130 1144
Pr4CLPromoter(1650bp) (618) ATTAATTCTTATTTTTTAATTTTTTTACATAGAAAATATCTTTA
SEQ ID NO 11 (1096) ATTAATTCTTATTTTTTAATTTTTTTACATAGAAAATATCTTTA
Consensus (1101) ATTAATTCTTATTTTTTAATTTTTTTACATAGAAAATATCTTTA
Section 27
(1145) 1145 1150 1160 1170 1188
Pr4CLPromoter(1650bp) (662) TATTATATACGAGAAATATAGAATGTTCTAGTCCAAGGACTATT
SEQ ID NO 11 (1140) TATTGTATCCAAGAAATATAGAATGTTCTCGTCCAGGGACTATT
Consensus (1145) TATT TAT C AGAAATATAGAATGTTCT GTCCA GGACTATT
Section 28
(1189) 1189 1200 1210 1220 1232
Pr4CLPromoter(1650bp) (706) AATTTCCAAATAAGTTTCAAAATCATTACATTAAAACTCATCAT
SEQ ID NO 11 (1184) AATCTCCAAACAAGTTTCAAAATCATTACATTAAAGCTCATCAT
Consensus (1189) AAT TCCAAA AAGTTTCAAAATCATTACATTAAA CTCATCAT
Section 29
(1233) 1233 1240 1250 1260 1276
Pr4CLPromoter(1650bp) (750) GTCATTTGTGGATTGGAAATTAGAC---AAAAGAGAA-------
SEQ ID NO 11 (1228) GTCATTTGTGGATTGGAAATTATATTGTATAAGAGAAATATAGA
Consensus (1233) GTCATTTGTGGATTGGAAATTA A A AAGAGAA
Section 30
(1277) 1277 1290 1300 1310 1320
Pr4CLPromoter(1650bp) (784) ----------TCC---CAAAT-------
SEQ ID NO 11 (1272) ATGTTCTCGTCTAGGGACTATTAATTTCCAAACAAATTTCAAAA
Consensus (1277) TCC CAAAT
Section 31
(1321) 1321 1330 1340 1350 1364
Pr4CLPromoter(1650bp) (792) --ATTTCTCTCAATCTC----------
SEQ ID NO 11 (1316) TCATTACATTAAAGCTCATCATGTCATTTGTGGATTGGAAATTA
Consensus (1321) ATT C T AA CTC
Section 32
(1365) 1365 1370 1380 1390 1408
Pr4CLPromoter(1650bp) (807) --CCAAAATAAACCTAATTA----------ATATA
SEQ ID NO 11 (1360) GACAAAAAAAATCCCAAATATTTCTCTCAATCTCCCAAAATATA
Consensus (1365) C AAAA AA CC AA TA ATATA


Section 33
(1409) 1409 1420 1430 1440 1452
Pr4CLPromoter(1650bp) (830) GTTCGAACTCCATATTTTTGGGAATTGAGAATTTTTCTACCCAA
SEQ ID NO 11 (1404) GTTCGAACTCCATATTTTTGGAAATTGAGAATTTTTTTACCCAA
Consensus (1409) GTTCGAACTCCATATTTTTGG AATTGAGAATTTTT TACCCAA
Section 34
(1453) 1453 1460 1470 1480 1496
Pr4CLPromoter(1650bp) (874) TAATATATTTTTTTTATACATTTTAGAGATTTTCCAGACATATT
SEQ ID NO 11 (1448) TAATATATTTTTTT-ATACATTTTAGAGATTTTCCAGACATATT
Consensus (1453) TAATATATTTTTTT ATACATTTTAGAGATTTTCCAGACATATT
Section 35
(1497) 1497 1510 1520 1530 1540
Pr4CLPromoter(1650bp) (918) TGCTCTGGGATTTATTGGAATGAAGGTTTGAGTAATGAAGGTTT
SEQ ID NO 11 (1491) TGCTCTGGGATTTATTGGAATGAAGGTT----------
Consensus (1497) TGCTCTGGGATTTATTGGAATGAAGGTT
Section 36
(1541) 1541 1550 1560 1570 1584
Pr4CLPromoter(1650bp) (962) GAGTTATAAACTTTCAGTAATGCAAGTATCTTCGGTTTTTGAAG
SEQ ID NO 11 (1519) GAGTTATAAACTTTCAGTAATCCAAGTATCTTCGGTTTTTGAAG
Consensus (1541) GAGTTATAAACTTTCAGTAATCCAAGTATCTTCGGTTTTTGAAG
Section 37
(1585) 1585 1590 1600 1610 1628
Pr4CLPromoter(1650bp) (1006) ATACTAAATCCATTATATAATAAAAACACATTTTAAACACCAAT
SEQ ID NO 11 (1563) ATACTAAATCCATTATATAATAAAAACACATTTTAAACACCAAT
Consensus (1585) ATACTAAATCCATTATATAATAAAAACACATTTTAAACACCAAT
Section 38
(1629) 1629 1640 1650 1660 1672
Pr4CLPromoter(1650bp) (1050) TTAATGGGATTTCAGATTTGTATCCCATGCTATTGGCTAAGCCA
SEQ ID NO 11 (1607) TTAATGGGATTTCAGATTTGTATCCCATGCTATTGGCTAAGGCA
Consensus (1629) TTAATGGGATTTCAGATTTGTATCCCATGCTATTGGCTAAG CA
Section 39
(1673) 1673 1680 1690 1700 1716
Pr4CLPromoter(1650bp) (1094) TTTTTCTTATTGTAATCTAACCAATTCCAATTTCCGCCCTGGTG
SEQ ID NO 11 (1651) TTTTTCTTATTGTAATCTAACCAATTCTAATTTCCACCCTGGTG
Consensus (1673) TTTTTCTTATTGTAATCTAACCAATTC AATTTCC CCCTGGTG
Section 40
(1717) 1717 1730 1740 1750 1760
Pr4CLPromoter(1650bp) (1138) TGAACTGACTGACAAATGCGGCCCGAAAACAGCGAATGAAATGT
SEQ ID NO 11 (1695) TGAACTGACTGACAAATGCGGTCCGAAAACAGCGAATGAAATGT
Consensus (1717) TGAACTGACTGACAAATGCGG CCGAAAACAGCGAATGAAATGT

Section 41

(1761) 1761 1770 1780 1790 1804

Pr4CLPromoter(1650bp) (1182) CTGGGTGATCGGTCAAACAAGCGGTGGGCGAGAGAACGCGGGTG

SEQ ID NO 11 (1739) CTGGGTGATCGGTCAAACAAGCGGTGGGCGAGAGAGCGCGGGTG

Consensus (1761) CTGGGTGATCGGTCAAACAAGCGGTGGGCGAGAGA CGCGGGTG

Section 42

(1805) 1805 1810 1820 1830 1848

Pr4CLPromoter(1650bp) (1226) TTGGCCTAGCCGGGATGGGGGTAGGTAGACGGCGTATTACCGGC

SEQ ID NO 11 (1783) TTGGCCTAGCCGGGATGGGGGTAGGTAGACGGCGTATTACCGGC

Consensus (1805) TTGGCCTAGCCGGGATGGGGGTAGGTAGACGGCGTATTACCGGC

Section 43

(1849) 1849 1860 1870 1880 1892

Pr4CLPromoter(1650bp) (1270) GAGTTGTCCGAATGGAGTTTTCGGGGTAGGTAGTAACGTAGACG

SEQ ID NO 11 (1827) GAGTTGTCCGAATGGAGTTTTCGGGGTAGGTAGTAACGTAGACG

Consensus (1849) GAGTTGTCCGAATGGAGTTTTCGGGGTAGGTAGTAACGTAGACG

Section 44

(1893) 1893 1900 1910 1920 1936

Pr4CLPromoter(1650bp) (1314) TCAATGGAAAAAGTCATAATCTCCGTCAAAAATCCAACCGCTCC

SEQ ID NO 11 (1871) TCAATGGAAAAAGTCATAATCTCCGTCAAAAATCCAACCGCTCC

Consensus (1893) TCAATGGAAAAAGTCATAATCTCCGTCAAAAATCCAACCGCTCC

Section 45

(1937) 1937 1950 1960 1970 1980

Pr4CLPromoter(1650bp) (1358) TTCACATCGCAGAGTTGGTGGCCACGGGACCCTCCACCCACTCA

SEQ ID NO 11 (1915) TTCACATCGCAGAGTTGGTGGCCACGGGACCCTCCACCCACTCA

Consensus (1937) TTCACATCGCAGAGTTGGTGGCCACGGGACCCTCCACCCACTCA

Section 46

(1981) 1981 1990 2000 2010 2024

Pr4CLPromoter(1650bp) (1402) CTCAATCGATCGCCTGCCGTGGTTGCCCATTATTCAACCATACG

SEQ ID NO 11 (1959) CTC----GATCGCCTGCCGTGGTTGCCCATTATTCAACCATACG

Consensus (1981) CTC GATCGCCTGCCGTGGTTGCCCATTATTCAACCATACG

Section 47

(2025) 2025 2030 2040 2050 2068

Pr4CLPromoter(1650bp) (1446) CCACTTGACTCTTCACCAACAATTCCAGGCCGGCTTTCGAGACA

SEQ ID NO 11 (1999) CCACTTGACTCTTCACCAACAATTCCAGGCCGGCTTTCTATACA

Consensus (2025) CCACTTGACTCTTCACCAACAATTCCAGGCCGGCTTTC A ACA

Section 48

(2069) 2069 2080 2090 2100 2112

Pr4CLPromoter(1650bp) (1490) ATGTACTGCACAGGAAAATCCAATATAAAAGGCCGGCCTCCGCT

SEQ ID NO 11 (2043) ATGTACTGCACAGGAAAATCCAATATAAAAAGCCGGCCTCTGCT

Consensus (2069) ATGTACTGCACAGGAAAATCCAATATAAAA GCCGGCCTC GCT

Section 49

(2113) 2113 2120 2130 2140 2156
Pr4CLPromoter(1650bp) (1534) TCCTTCTCAGTAGCCCCCAGCTCATTCAGTTCTTCCCACTGCAG
SEQ ID NO 11 (2087) TCCTTCTCAGTAGCCCCCAGCTCATTCAATTCTTCCCACTGCAG
Consensus (2113) TCCTTCTCAGTAGCCCCCAGCTCATTCA TTCTTCCCACTGCAG

Section 50

(2157) 2157 2170 2180 2190 2200
Pr4CLPromoter(1650bp) (1578) GCTACATTTGTCAGACACGTTTTCCGCCATTTTTCGCCTGTTTC
SEQ ID NO 11 (2131) GCTACATTTGTCAGACACGTTTTCCGCCATTTTTCGCCTGTTTC
Consensus (2157) GCTACATTTGTCAGACACGTTTTCCGCCATTTTTCGCCTGTTTC

Section 51

(2201) 2201 2210 2220 2230 2244
Pr4CLPromoter(1650bp) (1622) TGCGGAGAATTTGATCAGGTTACCCATGG----------
SEQ ID NO 11 (2175) TGCGGAGAATTTGATCAGGTTCGGATTGGGATTGAATCAATTGA
Consensus (2201) TGCGGAGAATTTGATCAGGTT TGG

Section 52

(2245) 2245 2250 2260 2277
Pr4CLPromoter(1650bp) (1651) ----------
SEQ ID NO 11 (2219) AAGGTTTTTATTTTCAGTATTTCGATCGCCATG
Consensus (2245)

EXHIBIT C

The Plant Journal (2005) **43**, 553–567 doi: 10.1111/j.1365-313X.2005.02480.x

*Eg*MYB2, a new transcriptional activator from *Eucalyptus* xylem, regulates secondary cell wall formation and lignin biosynthesis

Monica Goicoechea[1,†], Eric Lacombe[1,†], Sylvain Legay[1], Snjezana Mihaljevic[1], Philippe Rech[1], Alain Jauneau[1], Catherine Lapierre[2], Brigitte Pollet[2], Daniel Verhaegen[3], Nicole Chaubet-Gigot[1] and Jacqueline Grima-Pettenati[1,*]

[1]*Institut Fédératif de Recherches FR40, Unité mixte de Recherches 5546, Université Paul Sabatier–Centre National de la Recherche Scientifique, Pôle de Biotechnologie Végétale, 24 chemin de Borde Rouge, BP 17 Auzeville, 31326 Castanet-Tolosan, France,*

[2]*Laboratoire de Chimie Biologique, Unité mixte de Recherches 206, Institut National de la Recherche Agronomique-Institut National Agronomique, 78850 Thiverval-Grignon, France, and*

[3]*CIRAD Forêt, TA 10/C Baillarguet BP 5035, 34398 Montpellier Cédex 05, France*

Received 2 March 2005; revised 25 May 2005; accepted 26 May 2005.
*For correspondence (fax +33 562 193 502; e-mail grima@scsv.ups-tlse.fr).
†Both authors contributed equally to this work.

Summary

***Eg*MYB2, a member of a new subgroup of the R2R3 MYB family of transcription factors, was cloned from a library consisting of RNA from differentiating *Eucalyptus* xylem. *EgMYB2* maps to a unique locus on the *Eucalyptus grandis* linkage map and co-localizes with a quantitative trait locus (QTL) for lignin content. Recombinant *Eg*MYB2 protein was able to bind specifically the *cis*-regulatory regions of the promoters of two lignin biosynthetic genes, cinnamoyl-coenzyme A reductase (*CCR*) and cinnamyl alcohol dehydrogenase (*CAD*), which contain MYB consensus binding sites. *Eg*MYB2 was also able to regulate their transcription in both transient and stable expression assays. Transgenic tobacco plants over-expressing *EgMYB2* displayed phenotypic changes relative to wild-type plants, among which were a dramatic increase in secondary cell wall thickness, and an alteration of the lignin profiles. Transcript abundance of genes encoding enzymes specific to lignin biosynthesis was increased to varying extents according to the position of individual genes in the pathway, whereas core phenylpropanoid genes were not significantly affected. Together these results suggest a role for *Eg*MYB2 in the co-ordinated control of genes belonging to the monolignol-specific pathway, and therefore in the biosynthesis of lignin and the regulation of secondary cell wall formation.**

Keywords: MYB, transcription, xylem, lignin, secondary cell wall.

Introduction

Lignin is one of the major components of the secondary walls of xylem cells, allowing mechanical support and efficient conduction of water and solutes over long distances within the vascular system. In woody plant species, a large proportion of photosynthetically assimilated carbon is channelled to lignin synthesis and, as a consequence, lignified cell walls represent a major proportion of plant biomass and a huge reservoir of carbon stored within the polymers of lignocelluloses (Boudet *et al.*, 2003).

Lignin biosynthesis involves the phenylpropanoid pathway, which converts phenylalanine to *p*-coumaroyl coenzyme A (CoA), the precursor of a wide range of phenolic compounds. The enzymes involved in this short sequence are *L*-phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL) (Figure 1). The subsequent hydroxylation and methylation steps have recently been shown to occur at the level of hydroxycinnamic acid esters and their corresponding aldehydes and/or alcohols (Humphreys and Chapple, 2002). The most likely route for the production of monolignols probably involves enzymatic reactions catalysed by *p*-hydroxycinnamoyl CoA: quinate/shikimate *p*-hydroxycinnamoyl

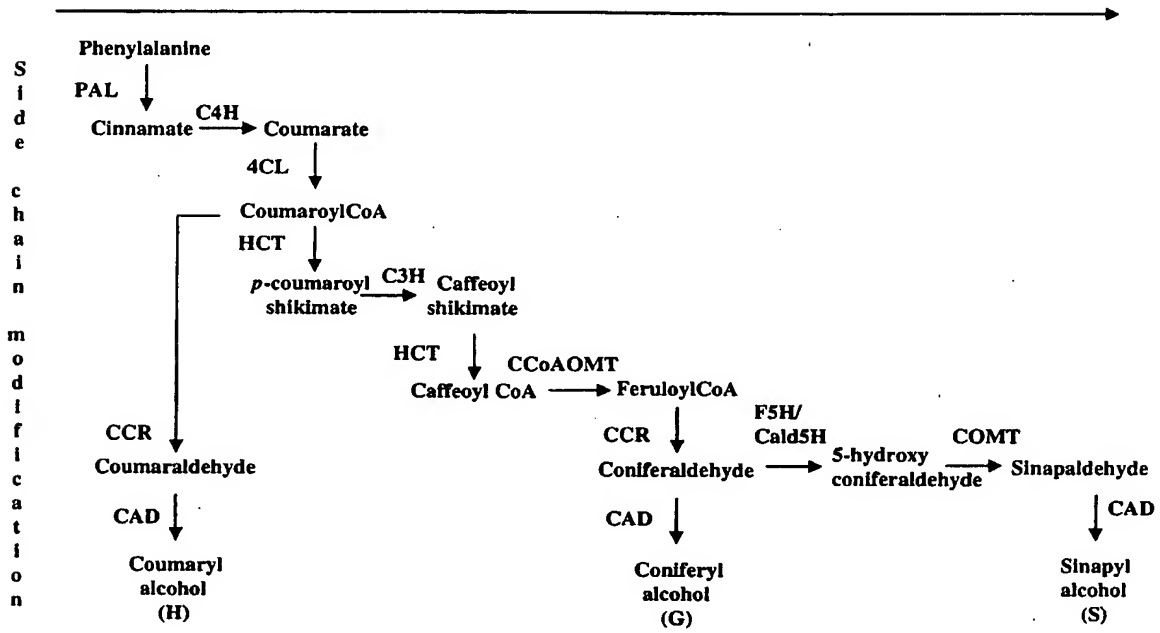


Figure 1. Model of monolignol biosynthesis pathway in angiosperms.
Enzymes involved in side-chain modification: PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate CoA ligase; HCT, *p*-hydroxycinnamoyl CoA: shikimate *p*-hydroxycinnamoyltransferase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase. SAD, sinapyl alcohol dehydrogenase. Enzymes involved in ring modification: C4H, cinnamate 4-hydroxylase; C3H, coumaroyl-quinate//shikimate 3-hydroxylase; CCoAOMT, caffeoyl CoA *O*-methyl transferase; F5H, ferulate 5-hydroxylase or Cald5H, coniferaldehyde-5-hydroxylase; COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase. The coumaryl, coniferyl and sinapyl alcohols are transported to the cell wall and polymerized to give rise to hydroxyphenyl (H), guaiacyl (G) and sinapyl (S) lignin, respectively.

transferase (HCT); coumaroyl-quinate/shikimate 3-hydroxylase (C3H); caffeoyl CoA *O*-methyltransferase (CCoAOMT); ferulate 5-hydroxylase (F5H, also called coniferaldehyde 5-hydroxylase, Cald5H); and caffeic acid *O*-methyltransferase (COMT) (for a review see Boerjan *et al.*, 2003) (Figure 1). Cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) catalyse the two last reductive steps leading to the three monolignols (*p*-coumaryl, coniferyl and syringyl alcohols), the monomeric units incorporated into the lignin heteropolymer.

In an attempt to gain insight into the mechanisms underlying the spatial and temporal control of lignification, we have characterized the genes encoding the terminal enzymes of lignin biosynthesis, CCR and CAD, and studied their expression during development. The promoters of the two genes from *Eucalyptus gunnii* (*EgCCR* and *EgCAD2*) direct expression in vascular tissues undergoing active lignification and preferentially in differentiating xylem (Feuillet *et al.*, 1995; Lacombe *et al.*, 2000; Lauvergeat *et al.*, 2002). The close correlation between *EgCCR* and *EgCAD2* promoter activities and lignification supports the view that tissue-specific transcription of these genes is a key step controlling the sites of lignin accumulation. Dissection of those promoters identified a *cis*-regulatory region of about 50–70 bp, responsible for driving this specific expression pattern in both promoters. The DNA–protein interaction sites have been mapped, and contain an AC-rich element corresponding to the MYB transcription factor binding consensus motif MBSIIG (MYB-binding site IIG, Lacombe *et al.*, 2000; Romero *et al.*, 1998; Sivadon and Grima-Pettenati, 2004).

The family of MYB transcription factors is one of the most abundant classes of transcription factors in plants, and the subfamily containing the two-repeat R2R3 DNA-binding domain is the largest (Stracke *et al.*, 2001). Some R2R3 MYB proteins bind AC elements found in the promoters of several genes of the phenylpropanoid pathway (Grotewold *et al.*, 1994; Sablowski *et al.*, 1994), and it has been suggested that these common motifs may provide a mechanism by which different steps of phenylpropanoid metabolism are co-ordinately regulated (Douglas, 1996; Martin and Paz-Ares, 1997; Weisshaar and Jenkins, 1998). Indeed, a number of R2R3 MYB proteins have been assigned functions in the regulation of phenylpropanoid biosynthesis and shown to regulate the biosynthesis of phenolic compounds, including lignin (Borevitz *et al.*, 2000; Jin *et al.*, 2000; Patzlaff *et al.*,

2003; Tamagnone *et al.*, 1998). Other transcription factors might also be involved (Rogers and Campbell, 2004).

As part of a programme aimed at characterizing 'lignin-specific' MYB *trans*-activators, we have cloned R2R3 MYB factors from a cDNA library of RNA from *Eucalyptus* differentiating xylem. Here we report the cloning and functional characterization of a new R2R3MYB gene, *EgMYB2*, which is able to bind the *EgCCR* and *EgCAD2* gene regulatory regions and to regulate their transcription. Transgenic tobacco plants over-expressing *EgMYB2* exhibited an increase in secondary wall thickness and an alteration in lignin composition. All the genes involved in the monolignol-specific biosynthesis pathway were upregulated, whereas the expression of core phenylpropanoid genes was not significantly affected. Taken together, the results reported here suggest that *Eg*MYB2 is a positive regulator of secondary cell wall formation and lignin biosynthesis.

Results

Eucalyptus Eg*MYB2 defines a new subgroup of the R2R3 MYB family*

To isolate MYB transcription factors potentially implicated in the regulation of lignin biosynthesis, we screened an *E. gunnii* xylem cDNA library (Lacombe *et al.*, 1997) with a MYB consensus sequence corresponding to the highly conserved amino acid sequence in the R3 repeat of the DNA-binding domain (Jackson *et al.*, 1991). Here we focus on the characterization of *EgMYB2* (AJ576023), a cDNA 1410 bp in length encoding a 321 amino acid protein (35.5 kDa, pI 4.92) which exhibits typical features of the R2R3 MYB protein family (Figure 2). The R2R3 DNA-binding domain comprises two imperfect repeats (54 and 51 amino acids, respectively), and has the predicted helix-turn-helix structures containing the conserved tryptophan residues involved in DNA binding (Martin and Paz-Ares, 1997). The predicted *Eg*MYB2 protein is closely related to other MYB proteins from different species within the R2R3 domain (Figure 2a,c) and shares the greatest homology with a *Populus trichocarpa* MYB protein *Poptr*1:49071 (86.4% similarity); and a MYB protein from Arabidopsis, *At*MYB83 (85.9% similarity; Romero *et al.*, 1998). Also closely related to *Eg*MYB2 are a MYB protein from a monocot species, *Hordeum vulgare* HvSPYMYB, *At*MYB46 and *Poptr*1:64485. A high level of homology was also found with a gymnosperm MYB protein, the pine *Pt*MYB4, which was recently shown to regulate lignin biosynthesis (Patzlaff *et al.*, 2003). *Eg*MYB2 belongs to group C as defined by Romero *et al.* (1998), most members of which bind preferentially to MBSIIG motifs G g/t T a/t GGT a/g.

The C-terminal domain of the *Eg*MYB2 protein does not contain any of the small conserved motifs used by Kranz *et al.* (1998) to classify MYB proteins in 22 subgroups. However, alignment of the C-terminal domain of *Eg*MYB2 with its closest related MYB proteins, *Poptr*1:49071 and *At*MYB83 (Figure 2b), revealed a conserved amino acid motif NX(R/K)(I/M)G(E/D)WDL(E/D)GL(M/I)(D/E)XXXSFPFLDF in the extreme C-terminal part of the protein. The presence of this C-terminal motif may define a new subgroup of MYB proteins, which possibly reflects the similar functions of its members.

EgMYB2 *maps to a unique locus on the* Eucalyptus grandis *linkage map and co-localizes with a QTL for lignin content*

The existence of a single *EgMYB2* gene in the *Eucalyptus* genome was supported by Southern hybridization at high stringency using the 3′ end as a probe (data not shown) and by genetic mapping. A full sib family of 201 interspecific hybrids between *Eucalyptus urophylla* and *E. grandis* was used to localize *EgMYB2* onto linkage maps previously constructed for both parents using RAPD markers (Verhaegen and Plomion, 1996; Verhaegen *et al.*, 1997). Using the single-strand conformation polymorphism (SSCP) technique (Orita *et al.*, 1989), *EgMYB2* was mapped on linkage group 2 of the *E. grandis* map (Figure 3). Interestingly, *EgMYB2* co-localizes with a quantitative trait locus (QTL) accounting for 4.5% of the variation in lignin content ($P = 0.009$). Although no linkage was found with the *E. urophylla* markers, a segregation (1:1:1:1) of the *EgMYB2* parental alleles was observed in the progeny. Using an ANOVA procedure between the four segregation classes, it was possible to discern a significant increase in *EgMYB2* maternal and paternal allele effects, explaining up to 7% of the variation of lignin content ($P = 0.005$).

EgMYB2 *is preferentially expressed in differentiating xylem tissue*

Using quantitative RT-PCR on *Eucalyptus* tissues, *EgMYB2* was shown to be preferentially expressed in the differentiating secondary xylem of stem and root, and in the central vein isolated from mature leaves (Figure 4). It is also expressed, albeit to a lesser extent, in the young part of stems and in young leaves containing developing veins. *EgMYB2* transcripts were present at very low levels in stem phloem, mature leaf blades, root bark and young lateral roots. This expression profile in lignin-rich tissue, which is in agreement with the fact that *Eg*MYB2 had been cloned from a *Eucalyptus* xylem cDNA library, could suggest that *Eg*MYB2 is involved in the regulation of lignin biosynthesis. Therefore we tested the effects of *Eg*MYB2 on two possible targets, the specific monolignol biosynthetic genes *EgCCR* and *EgCAD2*, which were also shown to be highly and preferentially expressed in *Eucalyptus* differentiating xylem (Grima-Pettenati *et al.*, 1993; Lacombe *et al.*, 1997).

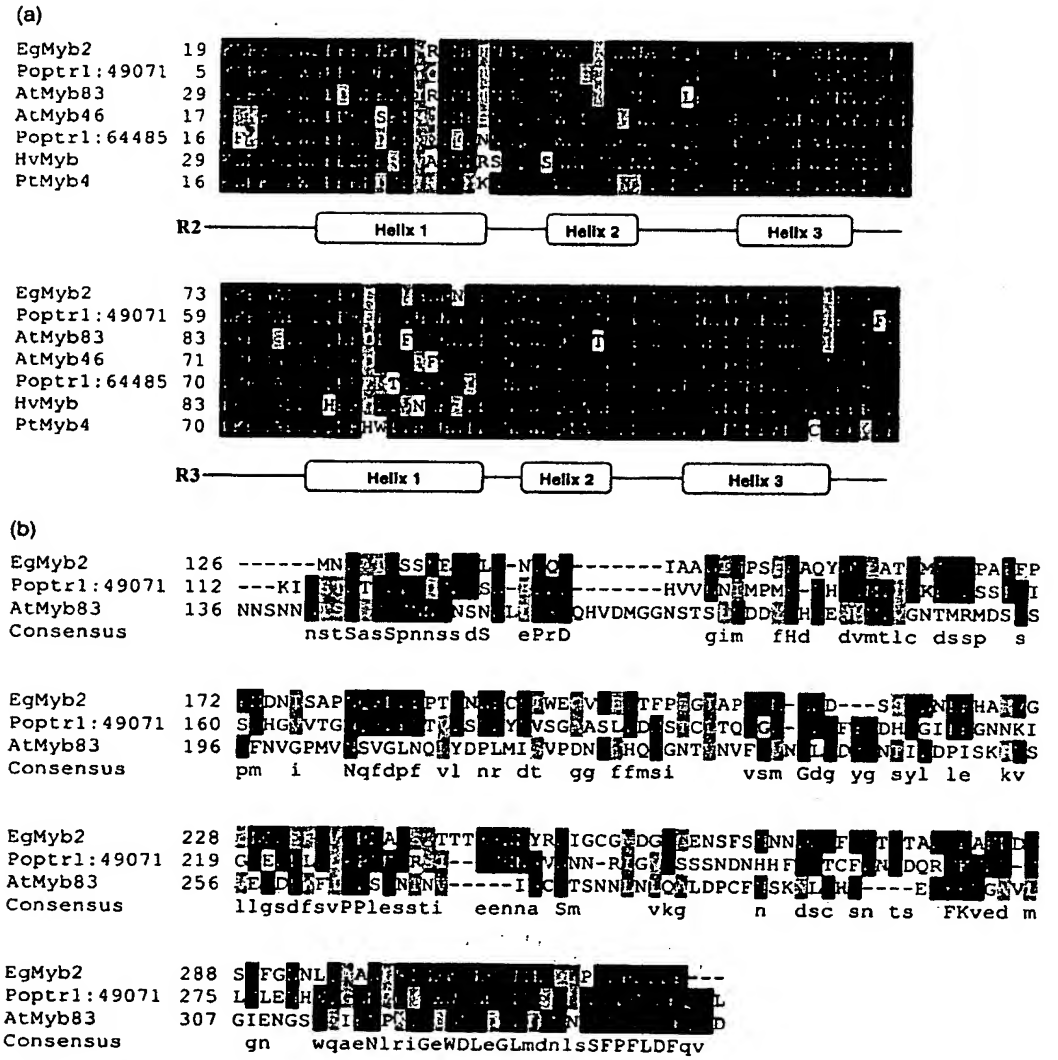


Figure 2. Sequence analysis of the *Eg*MYB2 protein.
(a) Amino-acid sequence alignment of the R2R3 domains of *Eg*MYB2 and other plant R2R3 MYB proteins. Multiple sequence alignments were generated using CLUSTALW (Thompson *et al.*, 1994). Residues highlighted in black are identical in more than 50% of the sequences; those highlighted in grey indicate conserved amino acid substitutions. The boxes below the alignment represent the predicted helix structure composing the two repeats.
(b) Alignment of the C-terminal regions of *Eg*MYB2 and its closest related MYB protein homologues. A conserved amino acid motif is remarkable in the extreme C-terminal region.
(c) Phylogenetic analysis of the R2R3 domain of *Eg*MYB2. Neighbour joining tree generated with MEGA 2.1 (Kumar *et al.*, 2001) using 1000 random sequence-addition bootstrap replication.
Genbank accession numbers: *Eg*MYB2 (AJ576023), *At*MYB46 (NM121290), *At*MYB83 (NM111685), *Pt*MYB4 (AY356371), *At*MYB4 (NM120023), *Hv*MYB3 (X70881), *Hv*SPYMYB (AY672068), *Le*MYB1 (X95297), *At*MYB61 (NP172425), *At*MYB55 (AF176000), *Dn*MYB2 (AF485893); *Poptr*1:49071 and *Poptr*1:64485 were found in the first draft of the *Populus trichocarpa* genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html).

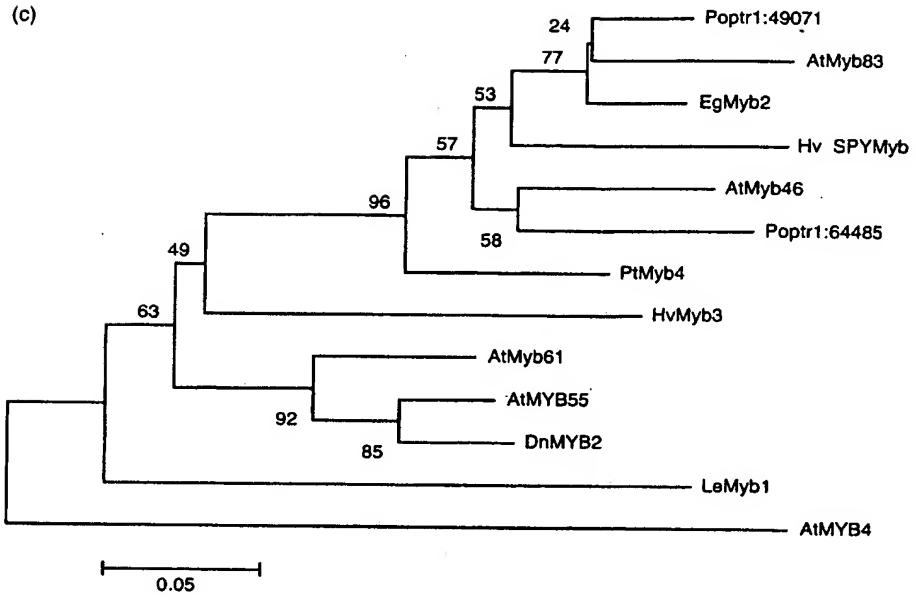


Figure 2. Continued.

Eg*MYB2 specifically binds the* cis*-regulatory regions of the* EgCCR *and* EgCAD2 *promoters*

As a first step to evaluate whether *Eg*MYB2 is involved in the transcriptional regulation of the *EgCCR* and *EgCAD2* genes, we tested the ability of *Eg*MYB2 to bind their *cis*-regulatory regions (Figure 5a) which contain MBSIIG sites (Figure 5b). For this purpose, the *EgMYB2* cDNA was fused to the glutathione-*S*-transferase coding sequence (*GST*) and expressed in *Escherichia coli* cells. Purified GST–*Eg*MYB2 protein was tested in electrophoretic mobility-shift assay (EMSA) for its ability to bind the cognate regulatory regions of *EgCAD2* (−203 to −129) and *EgCCR* (−119 to −70) promoters (Lacombe *et al.*, 2000; Lauvergeat *et al.*, 2002).

As shown in Figure 5(c), EMSA experiments revealed that *Eg*MYB2 is able to bind to both the *EgCAD2* (Figure 5c, lane 1) and *EgCCR* (Figure 5c, lane 5) promoter fragments. No DNA-binding activity was observed when using recombinant GST alone (data not shown), indicating that the interaction occurs specifically with EgMYB2. A 100-fold molar excess of unlabelled non-specific DNA fragment had no effect on the complex formation (Figure 5c, lanes 3 and 8), whereas effective competition was observed using either *EgCAD2* or *EgCCR* promoter fragments as specific competitors (Figure 5c, lanes 2 and 6). Cross-competition experiments have also been performed (Figure 5c, lanes 4 and 7), showing the ability of the *EgCAD2* and *EgCCR* promoter regions to compete reciprocally with the *Eg*MYB2 protein for binding. These gel-shift experiments show that recombinant *Eg*MYB2 is able to bind specifically *in vitro* to the regulatory regions of the *EgCCR* and *EgCAD2* promoters. These results raise the possibility that *Eg*MYB2 could control the co-ordinated expression of these two genes involved in the monolignol biosynthetic pathway.

Eg*MYB2 acts as a transcriptional activator of* EgCCR *and* EgCAD2 *promoters* in vivo

To test whether *Eg*MYB2 could transcriptionally regulate *EgCCR* and *EgCAD2* genes *in vivo*, the *EgCCR* and *EgCAD2* promoters fused to the GUS gene were used as reporter constructs (Lacombe *et al.*, 2000; Lauvergeat *et al.*, 2002) (Figure 6a). Each was co-transfected by *Agrobacterium* infiltration of *Nicotiana tabacum* (tobacco) leaves with an effector construct under the control of the 35S CaMV promoter, containing either the *EgMYB2* cDNA (referred to as *EgMyb2*$^+$), or the *EgMYB2* DNA-binding domain only (referred to as EgMyb2$^-$) (Figure 6a). Control values obtained using an effector construct without the *EgMYB2* gene (pJR1) exhibited significant levels of GUS activity. Interestingly, when *EgCCR* and *EgCAD2* promoters were co-transfected with *Eg*MYB2$^+$ construct, an induction of GUS activity was observed (Figure 6b). This increase was obtained reproducibly in several independent experiments, and was slightly higher for *EgCAD2* (2.4-fold) than for *EgCCR*

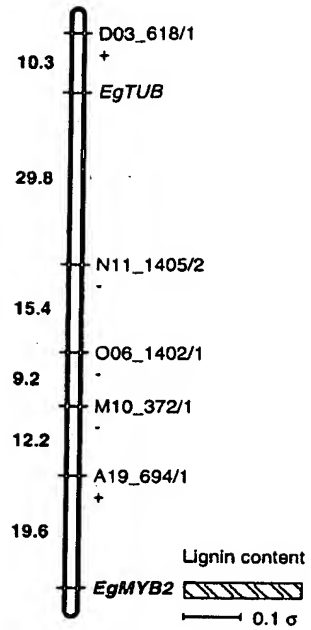


Figure 3. Genetic mapping of *EgMYB2* on *Eucalyptus* RAPD maps.
Using the single-strand conformation polymorphism (SSCP) technique, *EgMYB2* was located on *Eucalyptus grandis* linkage group 2 (for detailed maps see Verhaegen and Plomion, 1996). Distances along the linkage group are Kosambi centimorgans (cM); framework markers were ordered with an interval support ≥2. *EgMYB2* co-localizes with a QTL peak accounting for 4.5% of the phenotypic variation of the lignin content. *EgMYB2* effect is shown in cross-hatched bars expressed as phenotypic standard deviation (σ, difference between favourable QTL genotype and population mean).

(1.8-fold). Student's tests showed the relevance of these transactivation levels with significant values of $P < 0.001$ and $P < 0.04$ for transactivation of *EgCAD* and *EgCCR* promoters, respectively. The C-terminal region of *Eg*MYB2 appears to be the domain responsible for transcriptional activation of both promoters, as no activation was found using the *EgMYB2*$^-$ effector construct which contains only the DNA-binding domain.

Phenotypic changes induced by ectopic expression of Eg*MYB2 in transgenic tobacco*

To gain an insight into the role of *Eg*MYB2 *in planta*, we generated 18 independent transgenic tobacco plants expressing *EgMYB2* under the control of the 35S promoter (*EgMyb2*$^+$), and 15 dominant negative mutants expressing only the DNA-binding domain (*EgMyb2*$^-$).

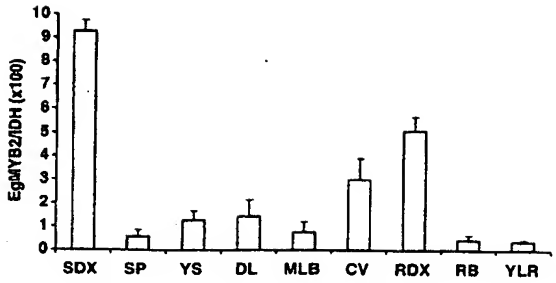


Figure 4. Expression of *EgMYB2* in *Eucalyptus* tissues.
Expression of *EgMYB2* was monitored by quantitative RT-PCR on RNA extracted from tissues of glasshouse-grown *Eucalyptus globulus*: stem differentiating secondary xylem, SDX; stem phloem, SP; young stems, YS; developing leaves, DL; mature leaf blades, MLB; central veins, CV; main root differentiating xylem, RDX; bark, RB; young lateral roots, YLR. Results are expressed as number of molecules (×100) relative to *Eg*IDH expression level as internal standard (see Experimental procedures). Two replicates were conducted using three independent biological individuals. Means and standard deviations are shown.

When grown *in vitro*, there were no obvious developmental differences among the primary transformants and control plants. After transfer to the glasshouse, *EgMyb2*$^-$ plants still showed no visible differences in growth and/or phenotypic aspect relative to control plants, whereas *EgMyb2*$^+$ plants exhibited a number of phenotypic differences (Figure 7). Half the plants transformed with the *EgMyb2*$^+$ construct (9/18) grew to only two-thirds of the size of the control plants (Figure 7a). Many of the *EgMyb2*$^+$ plants had two main stems emerging at the base of the plant (11/18) (Figure 7b), an effect generally associated with a loss of apical dominance. Interestingly, in hand-cut stem sections most plants also exhibited orange coloration of the xylem ring compared with the yellowish xylem of control plants (Figure 7c), probably reflecting an alteration of the secondary cell wall composition. Five independent primary transformants (2.7; 2.16; 2.18; 2.19; 2.24), all exhibiting orange-coloured xylem, were selected for study of segregation of the transgene in the progeny. Like most of the *EgMyb2*$^+$ plants they showed a significant reduction in seed production compared with controls, and the seeds were paler. Moreover, seeds of three transformants (2.18; 2.19; 2.24) were unable to germinate despite repeated attempts involving changes to the sterilization treatment, and even with no sterilization treatment at all. The percentage of germination of seeds from transformants 2.7 and 2.16 were 50 and 75%, respectively.

As a first step towards understanding why the seed did not germinate at all, or with a dramatically reduced efficiency, lignin staining was performed using phloroglucinol on seeds from control plants and transformants 2.7 and 2.18 (Figure 7d–f). Approximately half the seeds of transformant 2.7 stained more intensely than the control seeds, in

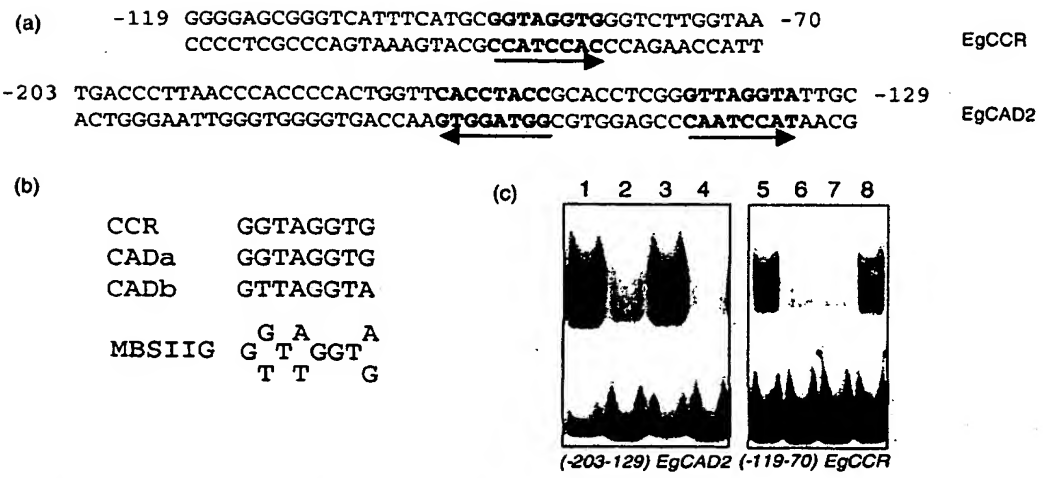


Figure 5. Binding of *Eg*MYB2 to the *cis*-regulatory regions of the *EgCAD2* and *EgCCR* promoters.
(a) Nucleotide sequences of *EgCAD2* and *EgCCR* promoter fragments used in electrophoretic mobility-shift assay (EMSA) experiments. These fragments correspond to the *cis*-regulatory regions involved in vascular expression. Positions given with respect to transcription start site. AC elements indicated in bold with arrows indicating their orientation.
(b) Nucleotide sequences of AC elements found in *EgCCR* and *EgCAD2* promoters compared with the MBSIIG consensus site (Romero *et al.*, 1998).
(c) EMSA in which the indicated radiolabelled promoter fragments were incubated with GST–*Eg*MYB2 recombinant protein in the absence (lanes 1, 5) or presence of 100× molar excess of non-specific (lanes 3, 8), [−203–129] *EgCAD2* (lanes 2, 7) or [−119–70] *EgCCR* (lanes 4, 6) unlabelled competitors. Amounts of GST–*Eg*MYB2 protein were 100 and 30 ng for *EgCAD2* and *EgCCR* promoter fragments, respectively. No DNA-binding activity was observed using recombinant GST alone (data not shown).

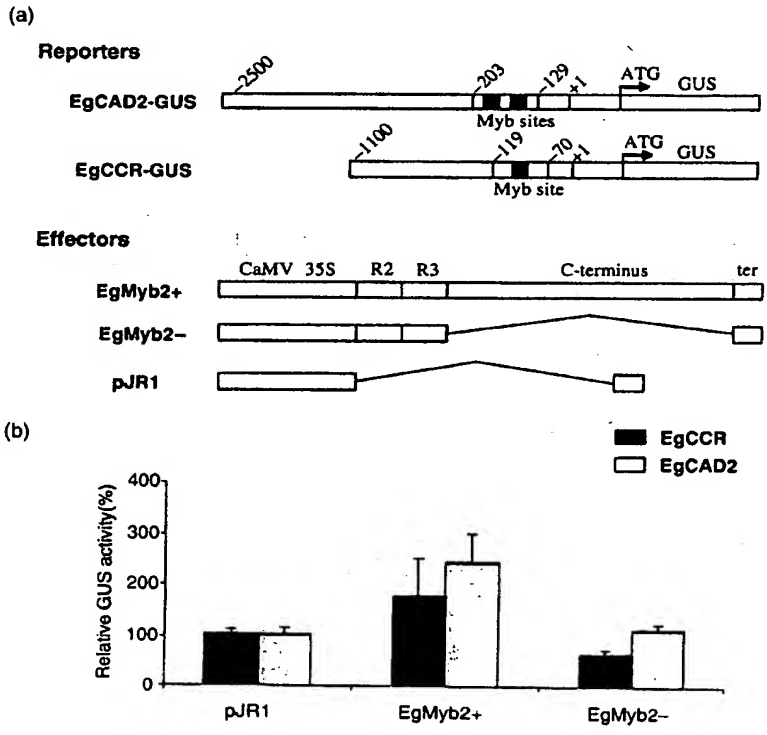


Figure 6. Effects of *Eg*MYB2 on transcriptional activities of the *EgCAD2* and *EgCCR* promoters *in vivo*.
(a) Schematic maps of the reporter and effector constructs. CaMV35S, 35S promoter; GUS, *uidA* coding region; (+1), transcription start site; R2R3, MYB DNA-binding domain; ter, nopaline synthase terminator.
(b) Results from co-transfection experiments in tobacco leaves. Agrobacteria containing effector and reporter constructs were co-infiltrated in tobacco leaves. Data represent mean values and standard deviations of three independent experiments, each containing at least three replicates. GUS activity is expressed as percentage of GUS activity relative to control (pJR1, 'empty' vector co-transfected with reporter constructs). Activations of *EgCAD* and *EgCCR* promoters are statistically significant relative to controls (Student's $P < 0.001$ and 0.04, respectively).

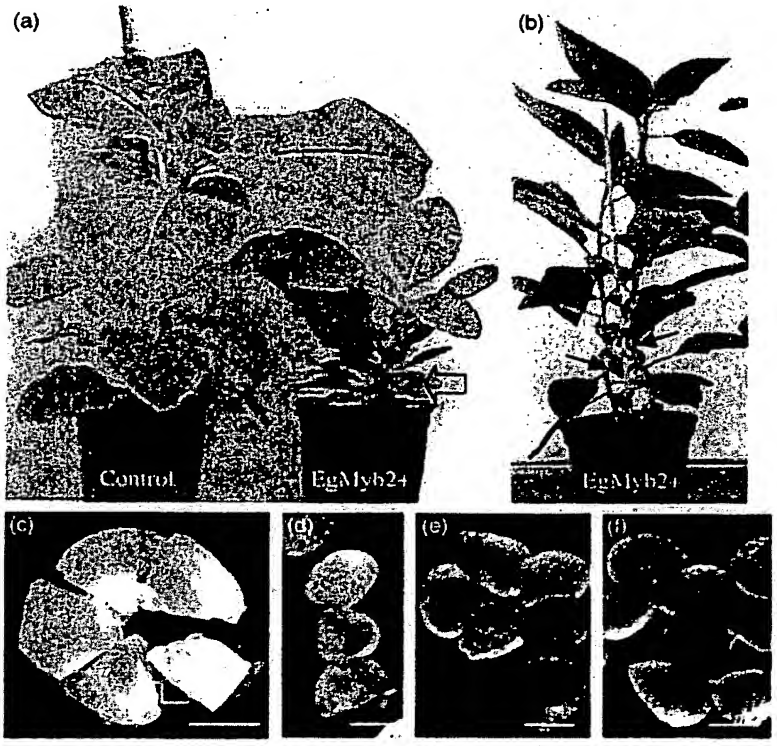


Figure 7. Phenotypic changes induced by *EgMYB2* ectopic expression in tobacco.
(a) Control plant and *EgMyb2*$^+$ primary transformant 2 weeks after transfer to the glasshouse.
(b) *EgMyb2*$^+$ primary transformant 6 weeks after transfer to the glasshouse. Note the presence of two main stems (arrows).
(c) Pattern of xylem coloration in hand-cut stem sections of four *EgMyb2*$^+$-independent primary transformants relative to control stem section (open arrow), scale 0.5 cm.
(d–f) Phloroglucinol staining of seeds (scale 0.8 mm): seeds of control (d); *EgMYB2*$^+$ primary transformant 2.7 (e); and transformant 2.18 (f).

agreement with the 50% germination rate (Figure 7e). All seeds of 2.18 which were unable to germinate stained more intensely than control seeds. Staining was concentrated in the region of the micropylar testa, where the radicle is known to emerge (Figure 7f).

The main phenotypic characteristics of primary transformants 2.7 and 2.16 (reduced plant size, loss of apical dominance, orange coloration of xylem) were associated with transgene expression, as these characteristics were maintained in the T_1 and T_2 progeny.

Eg*MYB2 increases xylem secondary cell wall thickness*

Cytological observations of xylem were performed on glasshouse-grown tobacco stem sections, either at low magnification using epifluorescence microscopy (Figure 8a,b) or at higher magnification using confocal microscopy (Figure 8c,d). At low magnification a significant increase in the number of lignified phloem fibres and xylem vessels was observed in some transformants, such as 2.18 (Figure 8b), compared with controls (Figure 8a).

Observations under confocal microscopy revealed that the xylem cell walls were thicker in the *Eg*MYB2$^+$ transformants (Figure 8d) compared with controls (Figure 8c). Measurements of fibre cell wall thickness indicate a dramatic thickening of xylem cell walls in transformant plants (4.9 ± 0.9 μm) compared with controls (3.5 ± 0.8 μm) (Figure 9). Student's test showed that the difference between the two populations is highly significant with $P < 0.001$ and $n = 500$. Observations of cell walls at the ultrastructural level by electron microscopy allowed us to demonstrate that the increase in cell wall thickness in *EgMyb2*$^+$ plants was due to an increase in the thickness of the middle S2 layer of the secondary cell wall (compare Figure 8g,h with controls in Figure 8e,f), whereas the S1 external and S3 internal layers appeared normal.

To gain information on the lignin content and composition within the xylem tissues, staining was performed using phloroglucinol reagent (Figure 8i,j); Maüle reagent (Figure 8k,l); and potassium permanganate for the visualization of lignin distribution (Figure 8m,n). The intensity of staining was always higher in transformed plants, whichever reagent was used, and appeared uniform across the thickened cell wall. The increase in reactivity of the cell walls using phloroglucinol and potassium permanganate could indicate a higher lignin content in xylem, which could be related to increased cell wall thickness. On the other hand, the strong reactivity to the Maüle reagent, which primarily stains syringyl lignin indicative of S units, suggests a higher S-unit composition in the lignin from transgenic lines.

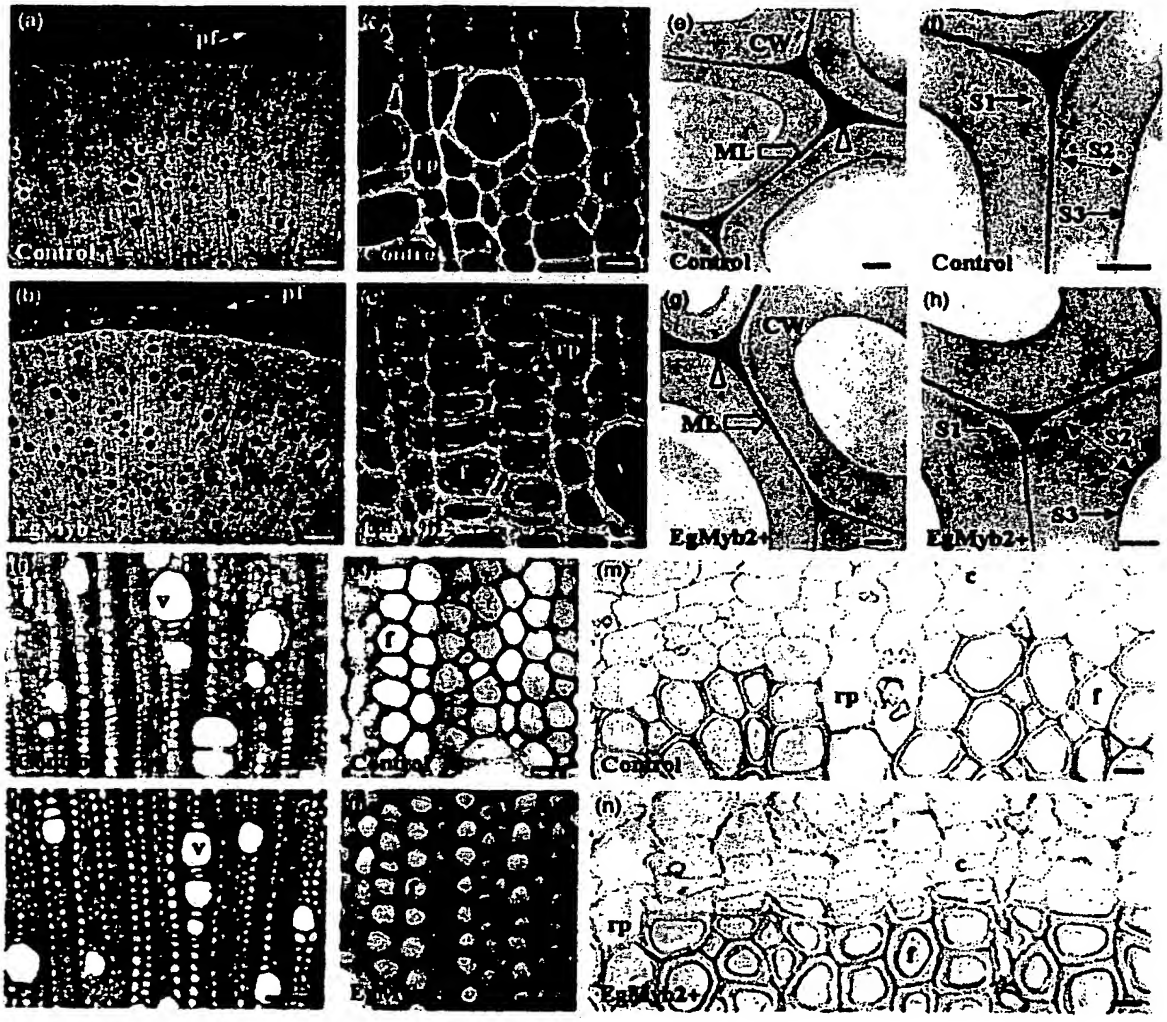


Figure 8. Cytological effects of *EgMYB2* over-expression in stem sections.
(a–d) Autofluorescence of xylem tissues on hand-cut stem sections under epifluorescence microscopy (a,b) and confocal microscopy (c,d) for control plant (a,c) and EgMYB2$^+$ transformant (b,d).
(e–h) Electron micrographs of $KMnO_4$-stained ultrathin stem sections of control (e,f) and *EgMyb2*$^+$ transformant (g,h).
(i–n) Phloroglucinol (i, j), Mäule (k,l) and potassium permanganate (m,n) stainings on transverse sections of control plants (i,k,m) and *EgMyb2*$^+$ transformant (j,l,n).
v, xylem vessel; rp, ray parenchyma; f, xylem fibres; pf, phloem fibres; c, cambial zone. S1–S3, layers within xylem fibre cell wall. Open arrows, middle lamella area (ML); arrowheads, cell junctions. Scale bars, 100 (a,b); 50 (i,j), 30 (k,l); 20 (c,d,m,n); 1 μm (e,f,g,h).

Eg*MYB2 controls the co-ordinated expression of genes involved in the lignin biosynthetic pathway*

The ability of *Eg*MYB2 to alter the expression of a complete set of genes involved in lignin biosynthesis (*PAL, C4H, 4CL, C3H, HCT, CCoAOMT, F5H, COMT, CCR, CAD*) was assessed by quantitative RT-PCR using RNA isolated from leaves of the transgenic lines described above. The assays were normalized to actin transcript levels. Figure 10 shows the relative levels of transcript accumulation found in glasshouse-grown *EgMyb2*$^+$ plants relative to control plants. Transcription of the two genes involved in the early steps of phenylpropanoid metabolism, *PAL* and *C4H*, was not significantly affected by the over-expression of *EgMYB2* and the third gene involved, *4CL*, appeared moderately (about threefold) activated. In marked contrast, all the genes

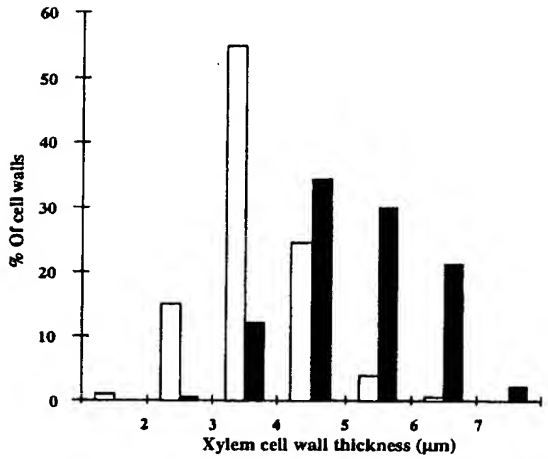


Figure 9. Effect of *EgMYB2* ectopic expression on the thickness of xylem fibres cell walls.
Frequency graph for comparison of cell wall thickness distribution (percentage of cell walls) between controls (open bars) and *EgMyb2*$^+$ plants (solid bars) within different classes. [0–2], thickness <2 µm; [2–3], thickness 2–3 µm, etc. 500 measurements were performed on semi-thin stem sections observed under bright light from two control plants and four independent primary transformants, using IMAGE-PRO PLUS software. Means and standard deviations, 4.9 ± 0.9 µm for *EgMyb2*+ plants; 3.5 ± 0.8 µm for controls. The difference between the two populations is highly significant (Student's $P < 0.001$).

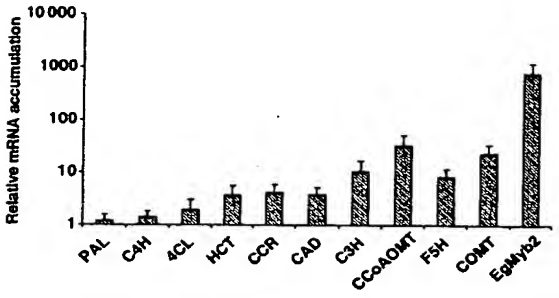


Figure 10. Quantitative RT-PCR analysis of transcript accumulation in transgenic tobacco plants over-expressing *EgMYB2*.
PAL, C4H, 4CL, C3H, HCT, COMT, F5H, CCoAOMT, CCR, CAD and *EgMYB2* mRNA accumulation was assessed by quantitative RT-PCR in leaves of four independent primary transformants. Transcript levels were normalized relative to the actin expression level as internal standard. Results expressed as mean and standard deviation relative to control plant the expression level of which has been assigned the value = 1 on the logarithmic scale.

encoding steps committed to monolignol biosynthesis were dramatically upregulated. Transcripts of the genes involved in conversion of esters to aldehydes, and aldehydes to alcohols (*HCT, CCR, CAD*) were about fivefold more abundant in transgenic *EgMyb2*$^+$ plants than in control plants, and transcript levels of the genes involved in the differential pathways leading to the three monolignol monomers (*C3H, CCoAOMT, F5H, COMT*) were up to 40-fold higher. Upregulation of the monolignol biosynthesis genes was also observed in stem tissue of the transgenic plants, but the extent was about twofold lower than in leaves, probably due to the fact that these genes are already activated by the endogenous tobacco MYB protein in stems.

Eg*MYB2 mainly controls the monomeric composition of lignin*

To test the effect of *Eg*MYB2 on lignin content and composition, we performed biochemical lignin determinations on the selected mature primary transformants. Despite the cytological observations and gene expression analysis described above, the data presented in Table 1 reveal that the Klason lignin content of the transformants does not differ substantially from the control level.

Lignin structure was investigated by thioacidolysis, a method that allows extraction of the lignin units involved in β-O-4 bonds (Lapierre *et al.*, 1986). The total yield of G and S lignin-derived main monomers recovered from the cell wall residue and referred to the Klason lignin was found to be similar in the transformants and control series. This result shows that the content of lignin units involved in uncondensed β-O-4 bonds is not significantly affected by the transgene. However, the S/G ratio was found to be significantly higher in the lignin extracted from *EgMyb2*$^+$ plants (1.27 ± 0.11 relative to control 1.09 ± 0.01, with Student's $P < 0.05$), essentially due to an increase in the amount of S units (Table 1). This result is consistent with the higher Maüle staining of the xylem samples from transgenic plants relative to the controls (Figure 8k,l). Interestingly, it is also related to the expression levels of the genes encoding CCoAOMT and COMT, enzymes involved in the methylation steps leading to the G and S monomeric units. Indeed, the highest S/G ratio in lignin (1.46, 34% higher than in control plants) was found in the transgenic plant in which the *COMT/CCoAOMT* expression ratio was highest (2.08). Conversely, the lowest S/G ratio among the transformants (1.18) was found in the plant in which the *COMT/CCoAOMT* expression ratio was lowest (0.16). The higher S/G value of the transformants relative to controls could be confirmed on T_2 plants (data not shown). Together, these data suggest that *Eg*MYB2 controls the *COMT/CCoAOMT* ratio which, in turn, has a direct consequence for the S/G monomeric ratio.

Discussion

As part of a programme aimed at identifying regulators of lignin biosynthesis, we have characterized a new R2R3 MYB

Table 1 Lignin analysis in *EgMyb2*$^+$ plants

Plant	Klason	S	G	S + G	S/G
2.7	20.91	919	749	1668	1.23
2.24	19.24	1019	867	1886	1.18
2.16	20.23	1070	854	1924	1.25
2.18	20.38	1077	881	1958	1.22
2.19	19.73	1163	798	1961	1.46
Mean *Eg*MYB2$^+$	20.10 ± 0.64	1049.6 ± 89.5	829.8 ± 55	1879 ± 122	1.27 ± 0.11
C06	19.02	953	874	1827	1.09
C30	19.7	1038	938	1976	1.1
C11	18.79	856	787	1643	1.09
Mean control	19.17 ± 0.47	949 ± 91	866.3 ± 75	1815 ± 166	1.09 ± 0.01

Five independent *Eg*MYB2$^+$ plants and three control plants were analysed for lignin content using the Klason method. Amount of lignin (referred to as Klason) is expressed as weight percentage of dried cell wall residue. Lignin composition was determined by thioacidolysis. Amounts of S and G monomers are expressed as μmol g^{-1} Klason lignin. Student's *t*-test showed that differences between controls and transgenics were significant with $P = 0.05$ for the S/G ratio. In contrast, the difference observed for the Klason lignin content does not appear as significant ($P = 0.07$).

gene (*EgMYB2*) from *Eucalyptus* that is highly and preferentially expressed in secondary xylem. This gene is single copy and maps to a QTL influencing lignin quantity. The *Eg*MYB2 protein is able to bind specifically the regulatory regions of the *EgCCR* and *EgCAD2* promoters *in vitro*, and to increase their transcription as shown by transient expression experiments. Together, these results raised the possibility that *Eg*MYB2 might control the co-ordinated expression of genes committed to the monolignol biosynthetic pathway. This hypothesis was further supported by transcript analysis of phenylpropanoid genes in transgenic tobacco plants over-expressing *EgMYB2*, which revealed significant increases in transcript abundance of genes known to be involved in the monolignol-specific portion of the pathway, but not those of the general phenylpropanoid pathway (*PAL, C4H*). The genes involved in conversion of esters to aldehydes, and of aldehydes to alcohols (side-chain modification), *HCT, CCR* and *CAD*, were about fivefold upregulated, whereas the genes involved in the differential pathways leading to the monolignol monomers (ring modification), *C3H, F5H, CCoAOMT* and *COMT*, were up to 40-fold upregulated.

As a consequence of the high increase in transcript abundance of the genes encoding enzymes involved in ring modification, the major main effect of *Eg*MYB2 was to alter the lignin monomeric composition. In angiosperms, the type of monomeric units within a lignin polymer depends on the degree of methylation of either the 3-hydroxyl groups or both 3-hydroxyl and 5-hydroxyl groups, leading to G and S units, respectively. CCoAOMT is believed to play an essential role in the synthesis of G units as well as in the supply of substrates for the synthesis of S units, whereas COMT essentially controls the biosynthesis of S units (Pincon *et al.*, 2001; Zhong *et al.*, 1998, 2000). In *EgMyb2*$^+$ plants we observed an increase in S/G ratio, mainly due to an increase in S units which probably results from the strong activation of *COMT*.

At the phenotypic level, plants over-expressing *Eg*MYB2 were characterized by reduced size in comparison with controls. With regard to this observation, it is interesting to draw a parallel with the recent findings of Kirst *et al.* (2004) showing that in an *E. grandis* back-cross family the genomic regions regulating growth are the same as those controlling lignin content and composition. Indeed, a negative correlation was found between transcript levels estimated for the lignin genes and growth. The most significant correlations were found for *F5H, C4H, C3H, COMT, CCoAOMT* and *CAD* (*CCR* was not analysed). It is worth noting that, as in the tobacco over-expressing *Eg*MYB2, S units were more abundant in slow-growing trees (38% increase) compared with fast-growing trees. It is possible that higher carbohydrate consumption for more lignin synthesis may have a negative effect on growth rate.

No significant increase in the Klason lignin content relative to the cell wall residue was detected in transgenic tobacco over-expressing *Eg*MYB2, in contrast to what could be expected from cytological analyses (intensity of phloroglucinol staining), and from the degree of activation of lignin biosynthetic genes. One plausible explanation for this apparent discrepancy relies on the very significant increase (40%) in thickening of the xylem cell walls, which has been observed whatever the method used for microscopic inspection. This thickening, assigned to the S2 layer of the secondary cell walls by electron microscopy, might be the consequence of an increase in the content of at least one of the other major constituents of secondary cell wall: cellulose and hemicelluloses, which would result in underestimating the increase in lignin content relative to cell wall residue.

Interestingly, an increase in phloroglucinol staining suggesting the presence of a higher lignin content or related condensed phenolics was observed not only in the walls of xylem cells, but also in the seed testa. In addition, the fact that the testa develops from the integuments of the ovule, and is therefore a maternal diploid tissue, provides an explanation for the complete absence of germination in the most extreme lines. It is likely that an alteration of cell wall composition occurred in the testa, probably preventing the action of hydrolases needed to complete germination and to allow the radicle to emerge.

At the protein level, the presence of a motif conserved in the C-terminal region of *Eg*MYB2 from *Eucalyptus*, *Poptr*1:4971 from cottonwood and *At*MYB83 from Arabidopsis may indicate functional similarity between these proteins and therefore define a new subgroup of MYB transcription factors. It is worth noting that neither the Arabidopsis MYB gene *At*MYB83 (nor its close sequence *At*MYB46) has been assigned any position among the subgroups defined in the Arabidopsis MYB family (Kranz *et al.*, 1998). This motif was not found in the C-terminal domain of *Pt*MYB4, a MYB gene isolated from a gymnosperm (*Pinus taeda*), highly expressed in xylem and involved in the regulation of lignification (Patzlaff *et al.*, 2003). When over-expressed in transgenic tobacco, both *EgMYB2* and *PtMYB4* act as transcriptional activators of genes committed to the lignin biosynthetic pathway, suggesting that their DNA-binding domains have similar selectivity. The increase in lignin content was higher in *PtMYB4* than in *EgMYB2* over-expressing plants. This might be due, at least in part, to the higher strength of the promoter used (double 35S CaMV) to direct *PtMYB4* expression compared with the single 35S CaMV used to drive *Eg*MYB2 expression. The relative weakness of the latter in xylem has been reported in other studies (Franke *et al.*, 2000). It should also be noted that ectopic expression of *PtMYB4* induces lignification in some cell types that normally do not lignify, such as cells from the pith. Such ectopic lignification was not observed in *EgMyb2*$^{+}$ plants, although in some transformants a higher number of lignified fibres were noticed. Although *Pt*MYB4 and *Eg*MYB2 share a number of common features, they also show some differences, suggesting that they are not functional homologues when working in their natural species. For instance, we have shown in *EgMyb2*$^{+}$ plants that among the most highly activated genes were those encoding enzymes responsible for control of the S/G ratio. Such a role would be unlikely for *Pt*MYB4, as lignins from pine do not contain S units.

Together, these results strongly suggest that *Eg*MYB2 is a positive regulator of secondary cell wall formation and lignin biosynthesis. Moreover, its co-localization with a QTL for lignin content renders it a good candidate for controlling lignin profiles that could be exploited in *Eucalyptus*-breeding programmes.

Experimental procedures

Recombinant DNA methods

Routine DNA methods were used according to Sambrook *et al.* (1989). DNA sequencing was performed with an ABI Prism 3700 DNA sequencer, using the ABI PRISM Dye terminator Cycle Sequencing Ready Reaction Kit (Amersham Pharmacia Biotech, Orsay, France).

cDNA library screening

Approximately 600 000 pfu of an amplified cDNA library from *E. gunnii* xylem in lambda ZAPII (Lacombe *et al.*, 1997) were blotted onto nitrocellulose membranes following the protocol recommended by the manufacturer (Stratagene, La Jolla, CA, USA), and screened using a consensus 38-mer oligonucleotide (5′-tkccmggaagracmgayaatgaaatcaagaaytaytgc) corresponding to a highly conserved motif in the R3 domain of MYB factors (PGRTDNEIKNYWN) (Jackson *et al.*, 1991), labelled with ^{32}P-ddATP (Amersham) and terminal deoxynucleotidyl transferase (Boehringer Mannheim, Meylan, France). Filters were hybridized overnight at 42°C in 5 × SSPE, 0.25% dry milk powder and 0.05% SDS. Washes were performed at 42°C in 2 × SSC, 0.1% SDS. Plaque-purified positive clones were converted into phagemids (pBluescript SKM13+) following Stratagene's instructions.

RNA isolation

Total RNA was extracted from various *Eucalyptus globulus* tissues harvested on 7-month-old glasshouse-grown plants as recommended by Southerton *et al.* (1998). Total RNA was extracted from leaves of mature glasshouse-grown wild-type and transgenic tobacco plants using the Extract-all kit (Eurobio, Paris, France). In both cases, total RNA was treated with Rnase-free Dnasel (Invitrogen, Cergy Pontoise, France) and purified on columns (Qiagen, RNA Mini Kit). RNA quality and quantity were checked by agarose gel and spectrophotometry.

Quantitative real-time RT-PCR

First-strand cDNA was synthesized from 1 μg RQ1 DNase-treated total RNA in a 20 μl reaction mixture containing 500 ng oligo dT_{12-18}, 0.5 mM dNTPs, 1 μl RNasin and 200 U SuperScript II reverse transcriptase, according to the manufacturer's instructions (Invitrogen). After incubation at 42°C for 1 h and at 65°C for 15 min, the cDNA was purified on Sephadex G50 columns in a 100 μl final volume. 2 μl cDNA was used as template in a quantitative real-time PCR assay (15 μl) performed on the LightCycler Instrument using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I reaction mix (Roche Applied Science, Meylan, France). After a initial denaturation step of 8 min at 95°C, 45 cycles of 15 sec at 95°C, 10 sec at 56–60°C (0–4°C below melting temperature, T_m) and 12 sec at 72°C were performed. Amplification specificity was checked by melting-curve analysis, and PCR efficiency was determined using standard curves constructed with serial dilutions of PCR products as templates. Actin was used as internal control for tobacco leaf samples. The amount of *EgIDH* transcript checked in different tissues of *Eucalyptus* exhibited cycle threshold values (C_t) of 20.13 cycles (mean) ± 0.51 (standard deviation), within the experimental error range of real-time PCR. Therefore *IDH* expression does appear constitutive in the different *Eucalyptus* tissues examined and was used as an internal

control. Quantification of expression ratios was performed according to the mathematical model developed by Pfaffl (2001). Primers and amplicon sizes: *Eucalyptus*, *EgMYB2* (152 bp; upper 5'-gcggatggagattctgtaca, lower 5'-aacgcccttccctactaaga); *EgIDH* (115 bp; upper 5'-ctgctggaatctggtatgaaca, lower 5'-tcactctggacatctccatca); *Tobacco* – in the case of multigene families primers have been designed to hybridize to all genes of the class postulated to have a role in lignin biosynthesis: *PAL* (94 bp, primers common to the two class I genes AB008200, X78269, upper 5'-gacaaagtgttcacagcaatg, lower 5'-taacagatwggaagaggagca); *C4H* (124 bp, D. Werck-Reichhart, IBMP, Strasbourg, France, personal communication, upper 5'-tcaacacaatggtggaatgc, lower 5'-actttgggacgtttggttca); *4CL* (89 bp, primers common to the two class I and class II genes U50845, U50846, upper 5'-cttctcaaccatcccaacatt, lower 5'-ctaacaacaaaagccactgga); *HCT* (127 bp, AJ507825, upper 5'-ggctgccaatccatgatgct, lower 5'-gcaacagattgactgccatca); *C3H* (112 bp, primers common to two very close genes; C. Chapple, Purdue University, IN, USA, personal communication, upper 5'-tggctgaggtgatcaagaac, lower 5'-tatgggaggttggggaagtc); *CCoAOMT* (96 bp, primers common to the four class I genes U38612, U62734, U62735, U62736, upper 5'-acaccctatggaatggatca, lower 5'-ccttgttgagttccaatacga); *F5H* (93 bp, primers common to two genes (unpublished data), upper 5'-gaaactctacgacttcaccc, lower 5'-tgactttgccggaatatggt]; *COMT* (132 bp, primers common to the two class I genes X74452, X74453, upper 5'-cctgcaaatgggaaggtgat, lower 5'-cagtcctttctttgcctcct); *CAD* (142 bp, primers common to the two very close genes X62343, X62344, upper 5'-ctcgggagaaagagcatcac, lower 5'-cctctccattgcagtgttga); *CCR* (139 bp, C. Halpin, University of Dundee, UK, personal communication, upper 5'-atgtgacgaagccaagggtaa, lower 5'-gtaggaattggaaggtgacct); *Actin* (139 bp, consensus primers to all tobacco constitutive actin genes, upper 5'-attgtkctcagtggtggctc, lower 5'-cctccaatccagacactgta).

Expression of GST-EgMYB2 in E. coli

The *EgMYB2* cDNA was recovered from the bluescript plasmid pBSK-*Eg*MYB2 using *Eco*RI and *Xho*I and directionally cloned into the expression vector pGEX-5X-1 (Amersham-Pharmacia). The in-frame fusion 3' to the *Glutathion S-Transferase* (*GST*) gene was checked by sequencing. The resulting plasmid was introduced in *E. coli* strain BL21 and induction of the GST–*Eg*MYB2 fusion protein was realized by adding isopropyl β-D-thiogalactoside (Sigma-Aldrich, St. Quentin, France) to a final concentration of 0.1 mM. After growth at 20°C for 6 h, cells were lysed in buffer: 20 mM Tris pH 7.5, 1 mM EDTA, 10% glycerol, 0,1% NP40, 100 mM PMSF, 10 μg ml^{-1} leupeptin; 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 2 mg ml^{-1} lysozyme, 5 U DNaseI (RQ1, Promega, Charbonnières, France). The fusion protein was purified from the soluble phase using glutathione–sepharose 4B matrix following the supplier's instructions (Amersham-Pharmacia). Protein concentration was estimated with Bradford reagent (Bio-Rad, Marnes la Coquette, France) and proteins extracts were analysed by SDS–PAGE and Western blotting using a mouse primary antibody anti-GST and a secondary antibody (IgG anti-anti-GST of mouse) conjugated with peroxidase.

Electrophoretic mobility shift assay

Vectors containing the *EgCAD2* (Feuillet *et al.*, 1995) or *EgCCR* (Lacombe *et al.*, 2000) promoters were used to amplify by PCR the [−203–129] *EgCAD2* regulatory fragment with the upper 5'-tctcgagatggctaaaaagcaagtcttgc-3' and lower primer 5'-ggcgaaaagtgacactcgagcaagc, and the [−119–70] *EgCCR* regulatory fragment with the upper primer 5'-ggtctcgagaggggagcg and the lower primer 5'-gactcgagttaccaaga, all the primers containing *Xho*I restriction sites. The PCR products were cloned in pGEM-T vector (Promega) and checked by sequencing. After *Xho*I digestion the resulting *EgCAD2* and *EgCCR* regulatory fragments were purified on agarose gels and 100 ng were 3'-end labelled for 30 min at 37°C with the Klenow fragment of DNA polymerase I (5 U) in a final volume of 20 μl with 33 μM of each of dATP, dTTP, dGTP and 4 μl α-^{32}P[dCTP] (10 mCi ml^{-1}). The labelled fragments were purified on a 4.8% polyacrylamide gel and eluted in water overnight at 4°C. Binding reactions were performed in a total volume of 25 μl, with 5000–10 000 cpm labelled DNA fragments (20–30 fmol), 30–100 ng purified GST–*Eg*MYB2 or GST alone, 10 mM Tris–HCl pH 8, 150 mM NaCl, 10% glycerol and 500 ng poly dIdC-poly dIdC (Gibco-BRL, Paris, France). For competition experiments, non-radioactive competitors were added to a 100-fold molar excess ratio relative to the probe. The binding reactions were incubated for 30 min at room temperature and analysed on a 4.8% polyacrylamide gel as previously described by Lacombe *et al.* (2000).

Binary constructs for transformation

The *EgMYB2* cDNA was subcloned as a *Kpn*I–*Xba*I fragment into the pGEM-T vector (Promega) and the sequence was checked. The 1.4 kb cDNA fragment was cloned into the binary vector pJR1 (Piquemal *et al.*, 1998) under the control of the 35S CaMV promoter, generating the *EgMyb2*$^{+}$ construct. For downregulation we generated a dominant negative construct. The plasmid pGEMT-*EgMYB2* (mentioned above) was digested with *Kpn*I and *Pst*I and the resulting 0.5 kb fragment corresponding to the DNA-binding domain (DBD) was placed under the control, the 35S *CaMV* promoter in an intermediary vector pBOB13 (EL, unpublished data). After digestion by *Hin*dIII and *Eco*RI, the [*35S promoter-EgMYB2 DBD-Nos terminator*] cassette was inserted into the binary vector pBin19, leading to the construct *Eg*MYB2^{-}. Using the freeze–thaw procedure (Holsters *et al.*, 1978), the constructs *EgMyb2*$^{+}$ and *EgMyb2*$^{-}$ were introduced into *Agrobacterium tumefaciens* strain LBA4404 for stable transformation, and/or into strain C581pCH3 for transient co-transfection experiments.

Co-transfection experiments

Co-transfection experiments were performed essentially according to the method of Yang *et al.* (2000). *Agrobacterium* strains C581pCH3, containing either a binary effector plasmid or a reporter construct, were co-infiltrated in near fully expanded leaves of tobacco plants using a 1 ml syringe. After agro-infiltration, plants were maintained in a growth chamber at 22°C under 16 h light for 3 days. Quantitative GUS assays were carried out on the infiltrated zone using 4-methylumbelliferyl-β-D-glucuronide as substrate (Jefferson, 1987). Protein concentrations were determined by the Bradford method (Bio-Rad). GUS activities were estimated as the mean of three independent assays, each containing at least three replicates.

Tobacco plant transformation

Tobacco (*Nicotiana tabacum* cv. Samsun NN) was transformed by a modification of the leaf-disc method (Horsch *et al.*, 1985). Regeneration and propagation procedures were as described by Piquemal *et al.* (1998). More than 15 independent tobacco transformants were generated for each construct, propagated *in vitro* and transferred to the glasshouse. The presence of the transgene was confirmed by PCR on genomic DNA using specific primers for kanamycin

resistance and for MYB genes (described above). F_1 seeds obtained by self-pollination of transformants were harvested and selected further on germination medium containing kanamycin (500 μg ml^{-1}). The sterilization treatment was for 2 min in ethanol 70% followed by 5 min NaOCl, 5%.

Microscopy and cell imaging

Transverse sections (100 μm thick) in the lower part of tobacco stems were obtained using a vibratome (Microcut H1250; Energy Beam Science Inc., St. Louis, MO, USA). They were observed either under UV excitation (excitation filter BP 340-380 nm, suppression filter LP 430 nm) or under bright-field after phloroglucinol (Wiesner reagent) and Maüle staining for lignin visualization. Other samples, dehydrated in ethanol, were embedded in Spurr's epoxy resin. Semi-thin (1–2 μm) and ultrathin sections (90 nm) were obtained using an ultramicrotome (Reichert UltraCutE; Leica Microsystems, Rueil Malmaison, France). Semi-thin sections, mounted on glass slides, were observed in confocal microscopy (LSM SP2; Leica) using the 488 nm ray line of the argon laser. The emitted light was collected between 500 and 550 nm. Other semi-thin and ultrathin sections were treated by $KMnO_4$ and observed either under bright-field or with an electron microscope at 80 kV (Hitachi, Naka, Japan). In optical microscopy, images were acquired using a CCD camera (Color CoolView; Photonic Science, Milham, UK). The thickness of the xylem cell walls was determined by image analysis (IMAGE PROPLUS software; Media Cybernetics, Silver Spring, MD, USA). Micrographs of seeds were acquired with a stereomicroscope (MZFLIII; Leica) equipped with a CDD camera (DC200; Leica).

Lignin analysis

Basal parts of stems of control and transgenic mature plants were harvested and frozen in liquid nitrogen. After lyophilization the stem samples were ball-milled to a fine powder and extracted as previously described by Piquemal *et al.* (1998) for subsequent determinations of Klason lignin contents using the Klason technique (Dence, 1992). Thioacidolysis was performed using the method of Lapierre *et al.* (1986).

Gene mapping and QTL analysis

Genetic mapping of *EgMYB2* was performed using an interspecific F_1 hybrid progeny (201 full sibs) between *E. urophylla* (female) and *E. grandis* (male) (Verhaegen and Plomion, 1996). Conformation polymorphism was detected using the SSCP technique as described previously by Gion *et al.* (2000) with specific primer pairs (upper 5′-tccaatccacaagacatagc, lower 5′-gtgggggaacagaaaactcg). In comparison with the *E. gunnii EgMYB2* sequence, four and six nucleotides were different in the *E. urophylla* and *E. grandis* sequences, respectively, indicating that both parents were heterozygous at this locus. Using a migration temperature of 15°C for 15 h electrophoresis, four bands were obtained, segregation of which in the progeny was consistent with the patterns observed in the parents. In order to map the newly genotyped SSCP *EgMYB2* marker to linkage maps previously established using RAPD markers for both parents (Verhaegen and Plomion, 1996; Verhaegen *et al.*, 1997), linkage analysis was performed using the MAPMAKER programme (Whitehead Institute, Cambridge, MA, USA) with a minimal linkage LOD of 6 and a maximum recombination fraction θ of 0.30. Assessments of lignin content using the Klason method was made on the 201 progenies used for establishing both genetic maps at 62 months, which corresponds to harvest age in commercial *Eucalyptus* plantations. The lignin content presented a normal distribution in the progeny studied. The QTL analysis was performed on each parental map under the back-cross model. Both the interval-mapping methods implemented in MAPMAKER/QTL (maximum likelihood) and QGENE (linear least squares; Whitehead Institute) with a threshold of 1.7 were used to declare a putative QTL for lignin content.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AJ576023.

Acknowledgements

The authors are grateful to Cathie Martin and C. Dubos for critical reading of the manuscript and useful suggestions. We wish to acknowledge Raphael Culerrier for assistance with real-time PCR, Yves Martinez and Amira El Far for assistance with microscopy, Colette Guez and Nathalie Ladouce for technical support, Frédéric Legée (INRA) for Klason lignin determination, Christine Boudet (CIRAD) for help with genetic mapping, and Clint Chapple (Purdue University) and Danielle Werck-Reichhart (IBMP, Strasbourg) for providing the sequences of the tobacco *C3H* and *C4H* cDNA clones, respectively. We wish to thank Pierre Sivadon and Etienne Paux for valuable discussions during the course of this work. This work was financially supported by the Conseil Régional Midi Pyrénées, the Université Paul Sabatier and the Centre National de la Recherche Scientifique. MG was the recipient of an EC postdoctoral fellowship. EL and PR were supported by grants from the Ministère de l'Enseignement Supérieur et de la Recherche.

References

Boerjan, W., Ralph, J. and Baucher, M. (2003) Lignin biosynthesis. *Annu. Rev. Plant Biol.* **54**, 519–546.

Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A. and Lamb, C. (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell*, **12**, 2383–2394.

Boudet, A.M., Kajita, S., Grima-Pettenati, J. and Goffner, D. (2003) Lignins and lignocellulosics: a better control of synthesis for new and improved uses. *Trends Plant Sci.* **8**, 576–581.

Dence, C.W. (1992) The determination of lignin. In *Methods in Lignin Chemistry* (Lin, S.Y. and Dence, C.W., eds). Berlin: Springer-Verlag, pp. 33–61.

Douglas, C.J. (1996) Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. *Trends Plant Sci.* **1**, 171–178.

Feuillet, C., Lauvergeat, V., Deswarte, C., Pilate, G., Boudet, A. and Grima-Pettenati, J. (1995) Tissue- and cell-specific expression of a cinnamyl alcohol dehydrogenase promoter in transgenic poplar plants. *Plant Mol. Biol.* **27**, 651–667.

Franke, R., McMichael, C.M., Meyer, K., Shirley, A.M., Cusumano, J.C. and Chapple, C. (2000) Modified lignin in tobacco and poplar plants over-expressing the *Arabidopsis* gene encoding ferulate 5-hydroxylase. *Plant J.* **22**, 223–234.

Gion, J.M., Rech, P., Grima-Pettenati, J., Verhaegen, D. and Plomion, C. (2000) Mapping candidate genes in *Eucalyptus* with emphasis on lignification genes. *Mol. Breed.* **6**, 441–449.

Grima-Pettenati, J., Feuillet, C., Goffner, D., Borderies, G. and Boudet, A.M. (1993) Molecular cloning and expression of a *Eucalyptus gunnii* cDNA clone encoding cinnamyl alcohol dehydrogenase. *Plant Mol. Biol.* **21**, 1085–1095.

Grotewold, E., Drummond, B.J., Bowen, B. and Peterson, T. (1994) The myb-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. *Cell*, **76**, 543–553.

Holsters, M., de Waele, D., Depicker, A., Messens, E., Van Montagu, M. and Schell, J. (1978) Transfection and transformations of *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* **163**, 181–187.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) A simple and general method for transferring genes into plants. *Science*, **227**, 1229–1231.

Humphreys, J.M. and Chapple, C. (2002) Rewriting the lignin roadmap. *Curr. Opin. Plant Biol.* **5**, 224–229.

Jackson, D., Culianez-Macia, F., Prescott, A.G., Roberts, K. and Martin, C. (1991) Expression patterns of *myb* genes from *Antirrhinum* flowers. *Plant Cell*, **3**, 115–125.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**, 387–405.

Jin, H., Cominelli, E., Bailey, P., Parr, A., Mehrtens, F., Jones, J., Tonelli, C., Weisshaar, B. and Martin, C. (2000) Transcriptional repression by *At*MYB4 controls production of UV-protecting sunscreens in *Arabidopsis*. *EMBO J.* **19**, 6150–6161.

Kirst, M., Myburg, A.A., De Leon, J.P.G., Kirst, M., Scott, J. and Sederoff, R. (2004) Coordinated genetic regulation of growth and lignin revealed by quantitative trait locus analysis of cDNA microarray data in an interspecific backcross of Eucalyptus. *Plant physiol.* **135**, 2368–2378.

Kranz, H.D., Denekamp, M., Greco, R. *et al.* (1998) Towards functional characterisation of the members of the R2R3-MYB gene family from *Arabidopsis thaliana*. *Plant J.* **16**, 263–276.

Kumar, S., Tamura, K., Jakobsen, I.B. and Nei, M. (2001) MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics*, **17**, 1244–1245.

Lacombe, E., Hawkins, S., Van Doorsselaere, J., Piquemal, J., Goffner, D., Poeydomenge, O., Boudet, A.M. and Grima-Pettenati, J. (1997) Cinnamoyl-CoA reductase, the first committed enzyme of the lignin branch biosynthetic pathway: cloning, expression and phylogenetic relationships. *Plant J.* **11**, 429–441.

Lacombe, E., Van Doorsselaere, J., Boerjan, W., Boudet, A.M. and Grima-Pettenati, J. (2000) Characterization of *cis*-elements required for vascular expression of the cinnamoyl-CoA reductase gene and for protein–DNA complex formation. *Plant J.* **23**, 663–676.

Lapierre, C., Rolando, C. and Monties, B. (1986) Thioacidolysis of poplar lignins: identification of monomeric syringyl products and characterisation of guaiacyl syringyl lignin fractions. *Holzforschung*, **40**, 113–118.

Lauvergeat, V., Rech, P., Jauneau, A., Guez, C., Coutos-Thevenot, P. and Grima-Pettenati, J. (2002) The vascular expression pattern directed by the *Eucalyptus gunnii* cinnamyl alcohol dehydrogenase *Eg*CAD2 promoter is conserved among woody and herbaceous plant species. *Plant Mol. Biol.* **50**, 497–509.

Martin, C. and Paz-Ares, J. (1997) MYB transcription factors in plants. *Trends Genet.* **13**, 67–73.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T. (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl Acad. Sci. USA*, **86**, 2766–2770.

Patzlaff, A., McInnis, S., Courtenay, A. *et al.* (2003) Characterisation of a pine MYB that regulates lignification. *Plant J.* **36**, 743–754.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, 2002–2007.

Pincon, G., Chabannes, M., Lapierre, C., Pollet, B., Ruel, K., Joseleau, J.P., Boudet, A.M. and Legrand, M. (2001) Simultaneous down-regulation of caffeic/5-hydroxy ferulic acid-*O*-methyltransferase I and cinnamoyl-coenzyme A reductase in the progeny from a cross between tobacco lines homozygous for each transgene. Consequences for plant development and lignin synthesis. *Plant Physiol.* **126**, 145–155.

Piquemal, J., Lapierre, C., Myton, K., O'Connell, A., Schuch, W., Grima-Pettenati, J. and Boudet, A.M. (1998) Down-regulation of cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. *Plant J.* **13**, 71–83.

Rogers, L.A. and Campbell, M.M. (2004) The genetic control of lignin deposition during plant growth and development. *New Phytol.* **164**, 17–30.

Romero, I., Fuertes, A., Benito, M.J., Malpica, J.M., Leyva, A. and Paz-Ares, J. (1998) More than 80 R2R3-MYB regulatory genes in the genome of *A. thaliana*. *Plant J.* **14**, 273–284.

Sablowski, R.W., Moyano, E., Culianez-Macia, F.A., Schuch, W., Martin, C. and Bevan, M. (1994) A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. *EMBO J.* **13**, 128–137.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Sivadon, P. and Grima-Pettenati, J. (2004) Transcriptional control of genes involved in lignin biosynthesis. In *Plantation Forest Biotechnology for the 21st Century* (Walter, C. and Carson, M., eds). Kerala, India: Research Signpost, pp. 203–218.

Southerton, S.G., Marshall, H., Moudarov, A. and Teasdale, R.D. (1998) Eucalypt MADS-Box genes expressed in developing flowers. *Plant Physiol.* **118**, 365–372.

Stracke, R., Werber, M. and Weisshaar, B. (2001) The R2R3-MYB gene family in *Arabidopsis thaliana*. *Curr. Opin. Plant Biol.* **4**, 447–456.

Tamagnone, L., Merida, A., Parr, A., Mackay, S., Culianez-Macia, F.A., Roberts, K. and Martin, C. (1998) The AmMYB308 and AmMYB330 transcription factors from *Antirrhinum* regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. *Plant Cell*, **10**, 135–154.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680.

Verhaegen, D. and Plomion, C. (1996) Genetic mapping in *E. urophylla* and *E. grandis* using RAPD markers. *Genome*, **39**, 1051–1061.

Verhaegen, D., Plomion, C., Gion, J.M., Poitel, M., Costa, P. and Kremer, A. (1997) Quantitative trait dissection analysis in *Eucalyptus* using RAPD markers: 1 Detection of QTL in interspecific hybrid progeny, stability of QTL expression across different ages. *Theor. Appl. Genet.* **95**, 597–608.

Weisshaar, B. and Jenkins, G.I. (1998) Phenylpropanoid biosynthesis and its regulation. *Curr. Opin. Plant Biol.* **1**, 251–257.

Yang, Y., Li, R. and Qi, M. (2000) *In vivo* analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. *Plant J.* **22**, 543–551.

Zhong, R., Ili, W.H., Negrel, J. and Ye, Z.H. (1998) Dual methylation pathways in lignin biosynthesis. *Plant Cell*, **10**, 2033–2046.

Zhong, R., Morrison, W.H., Ili, Himmelsbach, D.S., Poole, F.L., Ii and Ye, Z.H. (2000). Essential role of caffeoyl coenzyme A *O*-methyltransferase in lignin biosynthesis in woody poplar plants. *Plant Physiol.* **124**, 563–578.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☑ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.